



**Optimisation du traitement du cancer du sein
Triple-Négatif: développement des modèles de culture
cellulaire en trois dimensions, efficacité de l'Olaparib
(anti-PARP1) en combinaison avec la radiothérapie et
chimiorésistance instaurée par les protéines Multi Drug
Résistance**

Clémence Dubois

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Optimisation du traitement du cancer du sein Triple-Négatif : développement des modèles de culture cellulaire en trois dimensions, efficacité de l'Olaparib (anti-PARP1) en combinaison avec la radiothérapie et chimiorésistance instaurée par les protéines *Multi Drug Resistance*.

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Liste des abréviations

3D : Trois Dimensions

ABC : ATP Binding Cassette

ADN : Acide Désoxyribo Nucléique

ATP : Adénosine Tri-Phosphate

BCRP: Breast Cancer Resistance Protein

BER: Base Excision Repair

BL1/2: Basal Like 1/2

BRCA1/2: BREast Cancer genes 1 and 2

CDB: Cassure Double Brin

CDK: Cyclin Dependent Kinase

CSB : Cassure Simple Brin

DNA-PKcs : kinases ADN-dépendantes

EGF/EGFR: Epidermal Growth Factor

EHS: cellules Engelbreth, Holm and Swarm

ER (ER+/ ER-): Estrogen Receptor

FAC: 5-Fluorouracil, Adriamycin, Cyclophosphamide

FDA: Food and Drugs Administration

FEC: 5-Fluorouracil, Epirubicin, Cyclophosphamide

G2 : Growth 2 (phase du cycle cellulaire)

Gy: Gray

H2AX: Histone H2A member X

HER2: Human Epidermal growth factor Receptor 2

HR: Homologous Recombination

HRD: Homologous Recombination Deficient

IF: Impact Factor

IFP: Interstitial Fluid Pressure

IHC: ImmunoHistoChimie

IM: ImmunoModulatory

IMoST : Imagerie Moléculaire et Stratégies Théranostiques

IRM : Imagerie par Résonnance Magnétique

ITK : Inhibiteurs de Tyrosine Kinase

LAR : Luminal Androgen Receptor

LOT : Liquid Overlay Technique

MCS: Multi Cellular Spheroids

MDR: MultiDrug Resistance

MMR: MisMatch Repair

MRP1 (ABCC1): Multidrug Resistance Protein 1

MRP7 (ABCC10): Multidrug Resistance Protein 7

MSRO : Modulateur Sélectif des Récepteurs aux Oestrogènes

MXR: MultiXenobiotic Resistance

NBD: Nucleotid Binding Domain

NER: Nucleotide Excision Repair

NHEJ : Non Homologous End Joining (C-NHEJ pour canonique ou alt-NHEJ pour alternatif)

Ola-FL : Olaparib-FLuorescent

OMS : Organisation Mondiale de la Santé

P-gp : P-glycoprotéine

PARP : Poly-ADP-Ribose-Polymérase

PDMS : PolyDiMéthylSiloxane

PEG : PolyEthylène Glycol

Phase M : Phase de Mitose (phase du cycle cellulaire)

Phase S : Phase de Synthèse (phase du cycle cellulaire)

PR : Progesteron Receptor

Sous-type M : sous-type Mesenchymal

Sous-type MSL: Mesenchymal Stem Like

TEM : Transition Épithélio-Mésenchymateuse

TMDs : TransMembrane Domains

TN : Triple Négatif

TNM : Taille, Nodules, Métastases

VEGF: Vascular Endothelial Growth Factor

XRCC1: X-ray Repair Cross-Complementing protein 1

Glossaire

Acini : pluriel d'acinus, petite cavité glandulaire arrondie, se déversant dans un canal excréteur

Agents alkylants : molécules de chimiothérapie arrêtant la croissance des tumeurs en créant des ponts entre les bases guanine de l'ADN, empêchant la séparation des brins d'ADN et stoppant la division cellulaire

Agents antimitotiques : molécules de chimiothérapie ayant pour cible le fuseau mitotique ou le cytosquelette. Ces molécules favorisent la polymérisation, la dépolymérisation ou la dissolution des protéines du cytosquelette entraînant l'inhibition de la mitose

Agents intercalants : molécules de chimiothérapie s'intercalant, de par leur structure plane, entre les paires de bases d'ADN, rigidifiant celui-ci et entraînant l'apoptose

Amphipathiques : espèces chimiques possédant à la fois un groupement hydrophile et un groupement hydrophobe.

Antimétabolites : molécules de chimiothérapie inhibant certaines enzymes nécessaires dans le métabolisme cellulaire telle que la synthèse des purines ou de l'ADN polymérase, entraînant l'apoptose

Biomarqueurs : paramètres mesurables avec précision, utilisés comme indicateurs d'une fonction du corps, d'une maladie ou de l'action d'un médicament

BRCAness : phénotype correspondant à un déficit fonctionnel des protéines BRCA1/2 ou d'autres protéines impliquées dans le système de réparation des cassures double brin par recombinaison homologue, correspondant à un phénotype de cellules sensibles aux traitements par les anti-PARPs

Chimiothérapie : en Oncologie, désigne les traitements médicamenteux oraux ou injectables utilisés pour lutter contre le cancer

Chirurgie stéréotaxique : technique de neurochirurgie permettant de réaliser des biopsies ou des traitements dans des zones très précises du cerveau. Méthode beaucoup moins invasive que l'intervention de chirurgie classique.

Clonogénique : aptitude d'autoreproduction incontrôlée d'une cellule tumorale, capable de produire un nombre indéfini de clones, sous la forme de colonies

Culture cellulaire en 3D : technique de culture cellulaire *in vitro* permettant de recréer des microstructures dans un microenvironnement mimant plus fidèlement le comportement des tissus ou des tumeurs *in vivo*

Curage (ganglionnaire) : en Oncologie, opération chirurgicale consistant à extraire une partie d'un ganglion dans le but de l'analyser

Cytokératines : constituants des filaments intermédiaires de la kératine retrouvée dans les tissus épithéliaux. Les cytokératines constituent un marqueur histochimique de la différenciation des cellules épithéliales

Cytostatiques : propriété d'une molécule inhibant la croissance et la division cellulaire

Cytotoxiques : propriété d'une molécule induisant des effets délétères et finalement létaux pour la cellule

Épigénétique : discipline de la Biologie qui étudie la nature des mécanismes modifiant l'expression des gènes sans en modifier la séquence nucléotidique (ADN)

Flippase : enzyme intervenant dans le phénomène de bascule de certains phospholipides d'un feuillet à l'autre de la bicouche lipidique membranaire

Hormono-dépendance : phénomène propre à un tissu ou à une tumeur dont la croissance ou la prolifération sont stimulées par une hormone

Hormonothérapie ou **traitement antihormones** : en Oncologie, traitement consistant à inhiber l'action stimulante des hormones sur les cellules cancéreuses

Hypoxique : état d'oxygénation insuffisant de certains tissus

Index histopronostique : méthode de classification du degré d'agressivité des tumeurs par analyse histologique, considérant le degré d'anomalies nucléaires et cytoplasmiques, la différenciation cellulaire et le nombre de mitoses

Inhibiteurs de topoisomérase : molécules de chimiothérapie inhibant les enzymes assurant la spiralisation / dés spiralisation de l'ADN en créant des coupures transitoires de l'un ou des deux brins. Ces inhibiteurs empêchent la religation des coupures d'ADN, entraînant l'apoptose

Lactocytes : cellules glandulaires du tissu mammaire sécrétant le lait

Létalité synthétique : mort cellulaire résultant de la déficience simultanée de deux ou plusieurs gènes ou mécanismes, dont la déficience isolée n'a pas d'impact.

Matrice extracellulaire : ensemble de macromolécules présentes dans les tissus conjonctifs, facilitant les liaisons et l'adhérence intercellulaire

Matrigel® et **Geltrex®** : mélanges de protéines matricielles commercialisés sous une forme liquide à 4°C et gélifiant à température physiologique. Ces gels sont fréquemment utilisés pour la formation de cultures cellulaire en 3D

Microenvironnement : environnement immédiat d'une cellule ou d'un groupe de cellules

Mono ou co-cultures : cultures cellulaires en trois dimensions générées à partir d'un seul (mono) ou de plusieurs (co-cultures) types cellulaires

Monothérapie : traitement médicamenteux n'utilisant qu'un seul composé par cure

Oncogénétique : ensemble des techniques de Biologie Moléculaire visant à mettre en évidence les anomalies génétiques (mutations) induisant un risque héréditaire de prédisposition familiale à un ou plusieurs cancers

Organoïdes : amas cellulaires 3D reproduisant des micro-tissus ou micro-organes, obtenus à partir de cellules souches différenciées *in vitro*

Proto-oncogène : gène, souvent stimulateur de la croissance et la prolifération, dont l'activation est susceptible de conférer un phénotype cancéreux.

Scaffold : technique de formation des cultures cellulaires en 3D par l'injection des cellules au sein d'échafaudages cellulaires ou « scaffold », composés le plus souvent de bio polymères

Scaffold-free : technique de formation des cultures cellulaire en 3D n'utilisant pas d'échafaudages de bio polymères

Sites abasiques : emplacements de l'ADN où la base (purine ou pyrimidine) est manquante. Correspondent à un site « vacant » dans l'ADN.

Sphéroïdes : amas cellulaires 3D reproduisant des micro-tumeurs, obtenus à partir d'explants tumoraux ou de lignées cellulaires tumorales

Stades tumoraux : définissent le niveau d'extension d'une tumeur, du stade 0 (cancer localisé) au stade IV (cancer très invasif)

Suppresseurs de tumeurs (gènes) : gènes dont l'expression permet de limiter ou de ralentir le développement et la croissance tumorale et dont l'inactivation promeut le développement tumoral

Technique *Hanging drop* : technique de culture cellulaire en 3D utilisant des dispositifs spécifiques permettant de cultiver les cellules dans le fond d'une goutte de milieu suspendue

Technique *liquid overlay* : technique de culture cellulaire en 3D nécessitant l'usage de supports empêchant l'adhésion cellulaire et permettant l'agrégation cellulaire par gravité

Thérapies ciblées : traitements ayant pour objectif l'inhibition de la croissance ou de la propagation tumorale en ciblant des anomalies moléculaires et/ou des mécanismes promoteurs du développement et de la dissémination tumorale.

Traitement **Adjuvant** : traitement supplémentaire administré en complément de la chirurgie

Traitement **Néoadjuvant** : traitement administré afin de réduire la taille d'une tumeur avant le traitement de première intention (chirurgie ou radiothérapie)

Transition Épithélio-Mésenchymateuse (TEM) : désigne le passage d'un groupe de cellules épithéliales à une forme mésenchymateuse. Ce phénomène peut être réversible, les cellules subissant alors la Transition Mésenchymo-Épithéliale (TME)

Tumeurs « Triple-Négatives » : sous-type de tumeurs mammaires n'exprimant ou ne surexprimant pas les trois récepteurs hormonaux d'intérêt : récepteurs à la progestérone, aux oestrogènes et *Human Epidermal growth factor Receptor 2* (HER2)

Publications et communications issues de la thèse

Articles publiés:

1. **Dubois, C.**, Dufour, R., Daumar, P., Aubel, C., Szczepaniak, C., Blavignac, C., Mounetou, E., Penault-Llorca, F., Bamdad, M., 2017. *Development and cytotoxic response of two proliferative MDA-MB-231 and non-proliferative SUM1315 three-dimensional cell culture models of triple-negative basal-like breast cancer cell lines*. Oncotarget 8, 95316–95331. <https://doi.org/10.18632/oncotarget.20517>
2. Massard, C., **Dubois, C.**, Raspal, V., Daumar, P., Sibaud, Y., Mounetou, E., Bamdad, M., Awitor, O.K., 2017. *Cytotoxicity Study of Gold Nanoparticles on the Basal-Like Triple-Negative HCC-1937 Breast Cancer Cell Line*. J. Biomater. Nanobiotechnology 09, 13. <https://doi.org/10.4236/jbmb.2018.91002>
3. Daumar, P., Dufour, R., **Dubois, C.**, Penault-Llorca, F., Bamdad, M., Mounetou, E., 2018. *Development and validation of a high-performance liquid chromatography method for the quantitation of intracellular PARP inhibitor Olaparib in cancer cells*. J. Pharm. Biomed. Anal. 152, 74–80. <https://doi.org/10.1016/j.jpba.2018.01.036>

Articles rédigés :

1. **Clémence Dubois**, Fanny Martin *et al.* *Optimization of Triple-Negative breast cancer treatment: Modelization of a cotreatment combining the anti-PARP1 targeted therapy Olaparib® and fractioned radiotherapy on two and three-dimensional MDA-MB-231 and SUM1315 cell cultures.*
2. **Clémence Dubois**, Pierre Daumar *et al.* *Three-dimensional culture development of MDA-MB-231 and SUM1315 Triple-Negative Breast Cancer cell lines in the new synthetic serum-free medium OptiPASS.*
3. **Clémence Dubois**, Pierre Daumar *et al.* *Olaparib-FL development for the evaluation of anti-PARP penetration and distribution on 3D cell cultures of Triple-Negative breast cancer models.*
4. **Clémence Dubois**, Pierre Daumar *et al.* *MRP7 and P-gp chemoresistance proteins expression in MDA-MB-231 and SUM1315 spheroids following Olaparib treatment.*

Communications écrites :

1. **Poster** « Traitement combiné anti-PARP1 / radiothérapie : étude comparative de la survie d'une lignée de cancer du sein TN » - **Congrès SFRO (Paris), Octobre 2016** - **Dubois Clémence**, Martin Fanny, Daumar Pierre, Aubel Corinne, Donnarieix Denise, Maigne Lydia, Abrial Catherine, Prophète Lisa, Penault-Llorca Frédérique, Mounetou Emmanuelle & Bamdad Mahchid
2. **Poster** « *Development and cytotoxic response of 3D cell culture model of MDA-MB-231 « triple-negative basal-like » breast cancer cell line* ». **Congrès Cancéropole Lyon Auvergne Rhône Alpes CLARA (Lyon), Avril 2017** - **Dubois Clémence**, Dufour Robin, Daumar Pierre, Aubel Corinne, Sczepaniak Claire, Blavignac Christelle, Penault-Llorca Frédérique, Mounetou Emmanuelle & Bamdad Mahchid
3. **Poster** « *Development of 3D cell culture on Triple-Negative Breast Cancer cell lines: evaluation of Anti-PARP1 fluorescent agent distribution in spheroids* » **Congrès CRCL (Lyon), Septembre 2017** - **Clémence Dubois**, Pierre Daumar, Corinne Aubel, Frédérique Penault-Llorca, Emmanuelle Mounetou, Mahchid Bamdad
4. **Poster** « *Optistem synthetic medium: an adapted serum-free alternative for the three-dimensional cell culture of Triple-Negative breast cancer cell lines* ». **Congrès CLARA (Lyon), Avril 2018** - **Clémence Dubois**, Pierre Daumar, Corinne Aubel, Jean Gauthier, Pierre Vidalinc, Bernard Vidalinc, Emmanuelle Mounetou, Frédérique Penault-Llorca & Mahchid Bamdad

Communications orales :

1. **Communication Orale** « Développement de cultures cellulaires en trois dimensions à partir de lignées de cancer du sein Triple-Négatif : exemple d'utilisation de la fluorescence dans l'évaluation biologique de thérapies ciblées ». **Oncoriales (Saint Galmier), Octobre 2017. Prix de la meilleure présentation orale.**
2. **Communication Orale** « Optimisation du traitement du cancer du sein Triple-Négatif : modélisation d'un traitement combinant l'anti-PARP1 Olaparib et la radiothérapie fractionnée sur des lignées cellulaires cultivées en 2D et 3D ». **Journées de l'Ecole Doctorale (JED, Clermont-Ferrand), Juin 2018.**

Introduction générale

Le cancer du sein est un problème de santé publique majeur. En effet, malgré les efforts réalisés en termes de prévention et de dépistage, cette pathologie est une des premières causes de mortalité chez la femme. Parmi les différents sous-types de tumeurs mammaires, le modèle Triple-Négatif (TN) est particulièrement agressif et de mauvais pronostic. Afin d'améliorer les stratégies de prise en charge de ces tumeurs, de nouvelles thérapies ciblées ont été développées telles que les inhibiteurs de la Poly-ADP-Ribose-Polymérase 1 (inhibiteurs de PARP1). Ceux-ci ont montré des résultats prometteurs dans les tests précliniques et cliniques, néanmoins des résistances à l'échelle moléculaire et cellulaire ont été mises en évidence, qui représentent un frein majeur pour l'efficacité du traitement. Les tumeurs mammaires TN représentent l'un des principaux modèles d'étude du groupe de recherche préclinique « *Résistance* » de l'UMR INSERM 1240 IMoST (équipe 2). Celui-ci a pour objectif l'évaluation de l'efficacité des thérapeutiques ciblées, et notamment de l'action des anti-PARPs par modélisation *in vitro*. Afin d'optimiser le traitement de ces tumeurs TN, l'équipe étudie particulièrement le devenir intracellulaire des chimiothérapeutiques en corrélation avec les mécanismes de chimiorésistance, déjà présents et/ou instaurés par des protéines « *Multi Drug Resistance* » (MDR). Ces explorations étaient jusqu'à présent réalisées à partir de cultures cellulaires en deux dimensions. Néanmoins, celles-ci ne reflètent pas la conformation des cellules tumorales *in vivo*. Ainsi, d'autres méthodologies de culture cellulaire et notamment celles en trois dimensions (3D) sont de plus en plus développées comme outil de diagnostic pour étudier le comportement des molécules chimiothérapeutiques et la sensibilité des cellules tumorales.

Dans ce contexte, ces travaux de thèse ont porté sur le développement et la caractérisation des cultures cellulaires en 3D à partir de différentes lignées cellulaires de tumeurs TN. Par la suite, afin d'augmenter la reproductibilité des cultures cellulaires 3D, un milieu de culture synthétique sans sérum OptiPASS (BIPOPASS) a été adapté à ces cultures. Ensuite, sur ces modèles 2D et les sphéroïdes optimisés issus des lignées cellulaires TN, l'efficacité d'un co-traitement combinant l'Olaparib (anti-PARP1) et la radiothérapie fractionnée a été étudiée. Parallèlement, le développement d'un outils diagnostic fluorescent dérivé de l'Olaparib a permis d'étudier le degré de pénétration/d'efficacité/de cytotoxicité des anti-PARPs sur ces modèles de cultures cellulaires 3D. Par ailleurs, la caractérisation de la

chimiorésistance induite par les protéines de *MultiDrug Resistance* MRP7 et P-gp suite au traitement par l'Olaparib a été réalisée sur les modèles 2D et 3D. Finalement, dans le cadre d'une collaboration, l'étude de la toxicité de nanoparticules d'or PEGylées a été réalisée sur une lignée de tumeur TN.

Synthèse bibliographique

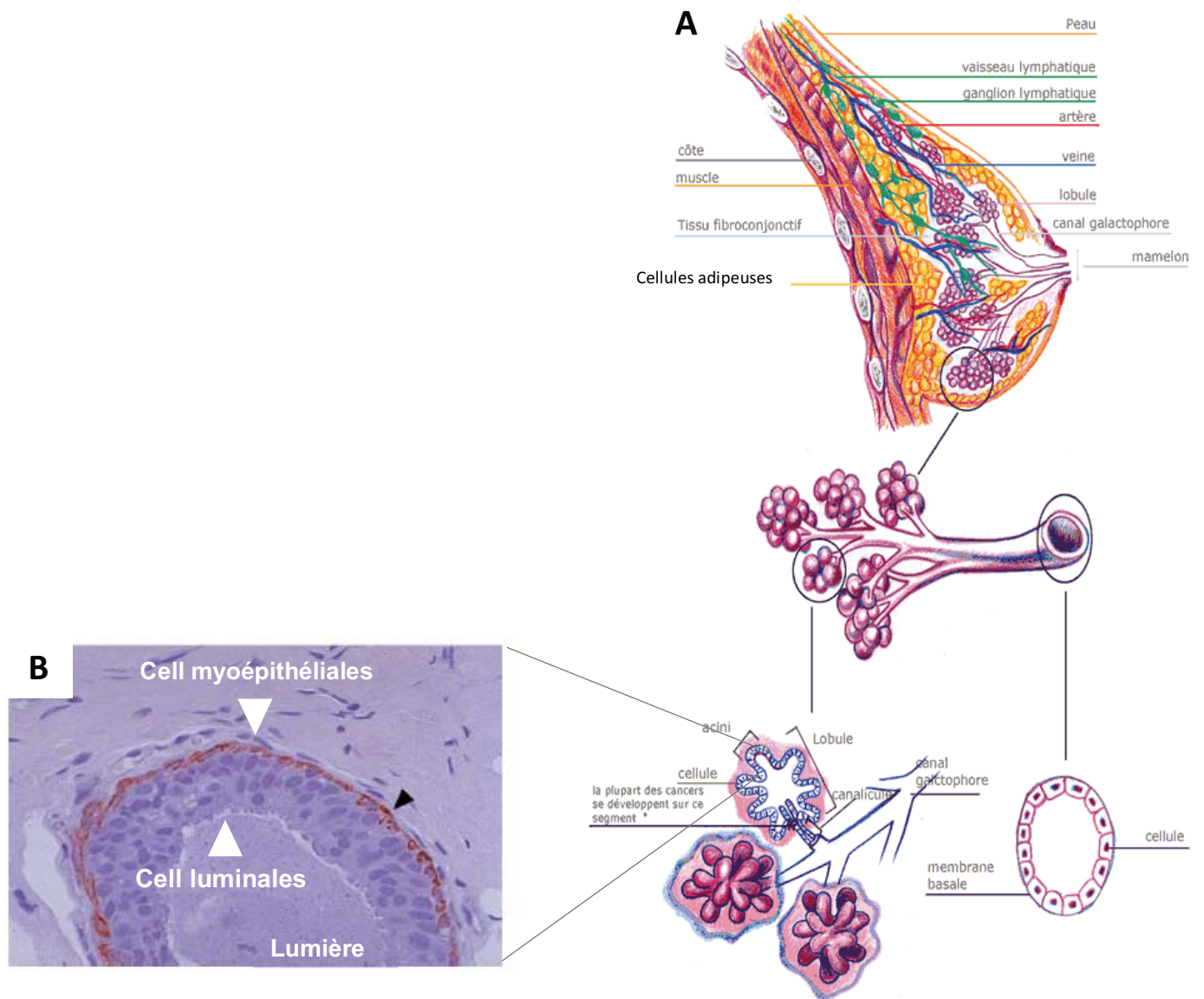


Figure 1 : Description anatomique du sein

- (A) Le sein est constitué de lobes, divisés en lobules, chacun contenant des acini. Les lobules sont ramifiés par des canaux galactophores permettant le transport du lait jusqu'au mamelon (**Jesinger, 2013**).
- (B) Description d'un acinus (G=40X) composé de cellules luminales sécrétrices entourées d'un anneau de cellules myoépithéliales contractiles permettant l'expulsion du lait *via* la lumière du tube (**Jesinger, 2013**).

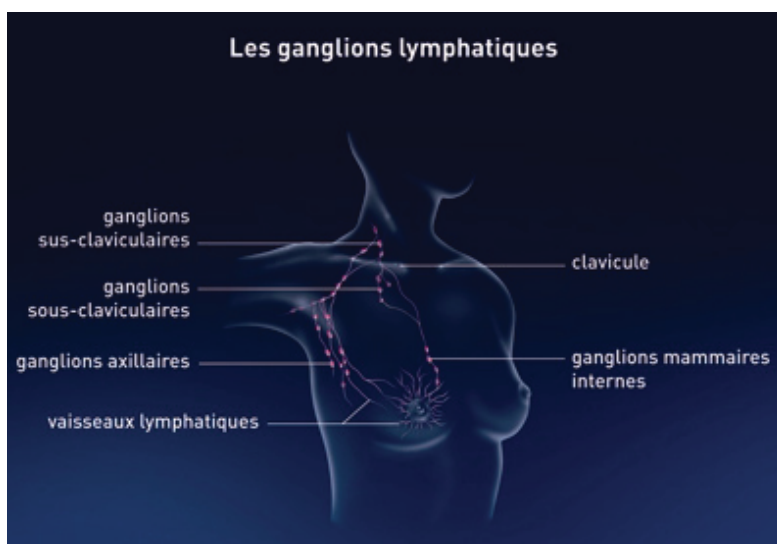


Figure 2 : Schéma lymphatique du sein - Le sein est parcouru de vaisseaux sanguins et lymphatiques (**Institut National du Cancer, Cancer du sein, Anatomie du sein**)

A) Les cancers du sein

I) Physiologie du tissu mammaire

Le sein est un organe glandulaire divisé en quinze à vingt lobes, chaque lobe comprenant de nombreux lobules, eux-mêmes composés d'acini* (**Figure 1**). Ces derniers sont les lieux de production et de stockage du lait. Ils sont constitués de cellules épithéliales sécrétrices appelés lactocytes*, organisées en couches basales et luminales. Les cellules luminales sont proches de la lumière du tube, et expriment les cytokératines* CK8/18 et 19. La couche basale est quant à elle constituée de cellules myoépithéliales à capacité contractile permettant l'expulsion du lait et exprimant les cytokératines CK5/6, 14 et 17 (**Figure 1B**). Celles-ci produisent également des protéines de la matrice extracellulaire*, et notamment la laminine 11, influençant la polarité et la survie des cellules luminales (**M. Simian et al. 2001**). Les acini sont organisés autour d'un canal excréteur commun, appelé canal galactophore (**Figure 1A**). Ces canaux se ramifient au sein de chaque lobule et lobe, afin d'acheminer le lait jusqu'aux mamelons. Le tissu mammaire est également constitué de cellules adipeuses, fibroblastiques, immunitaires, et vasculaires (**Inman et al. 2015**). Les cellules adipeuses (**Figure 1A**) forment la majorité du stroma mammaire, qui constitue un réservoir de lipides nécessaire au processus très énergivore de production de lait. Ces adipocytes ont également un rôle endocrine de régulation de la croissance et de la fonction des cellules épithéliales sécrétrices (**Russell C. Hovey et Aimo 2010**). Par ailleurs, les adipocytes produisent certains facteurs régulant l'angiogenèse de la glande mammaire (VEGF) (**R. C. Hovey et al. 2001**). Les fibroblastes, englobés dans le tissu adipeux et fréquemment retrouvés à proximité des cellules basales, ont un rôle dans la survie des cellules épithéliales et dans la morphogénèse du tissu adipeux (**Xiuli Wang et Kaplan 2012**). De plus, ces cellules produisent la matrice extracellulaire mammaire et notamment le collagène, les protéoglycanes et la fibronectine. Les fibroblastes synthétisent également des métalloprotéases, enzymes de dégradation de la matrice extracellulaire, permettant la libération et le passage de certains facteurs de croissance ou de cytokines (**Visvader et Stingl 2014**). Le tissu mammaire se développe grâce à deux hormones principales produites par les ovaires, que sont l'œstrogène et la progestérone. Ces deux hormones, se fixant sur leurs récepteurs spécifiques (les récepteurs aux oestrogènes **ER** et à



Hippocrates (460-370 AJC) « Père de la médecine »



Saint Galen (131-203) : la théorie humorale



René Descartes (1596-1650) : la théorie lymphatique



Henri François le Dran (1685-1770) : la théorie « métastatique »



William Halsted (1852-1922) : Père de la chirurgie en Amérique, première mastectomie radicale

Figure 3 : Principales figures du cancer à travers les siècles (Lukong, 2017)

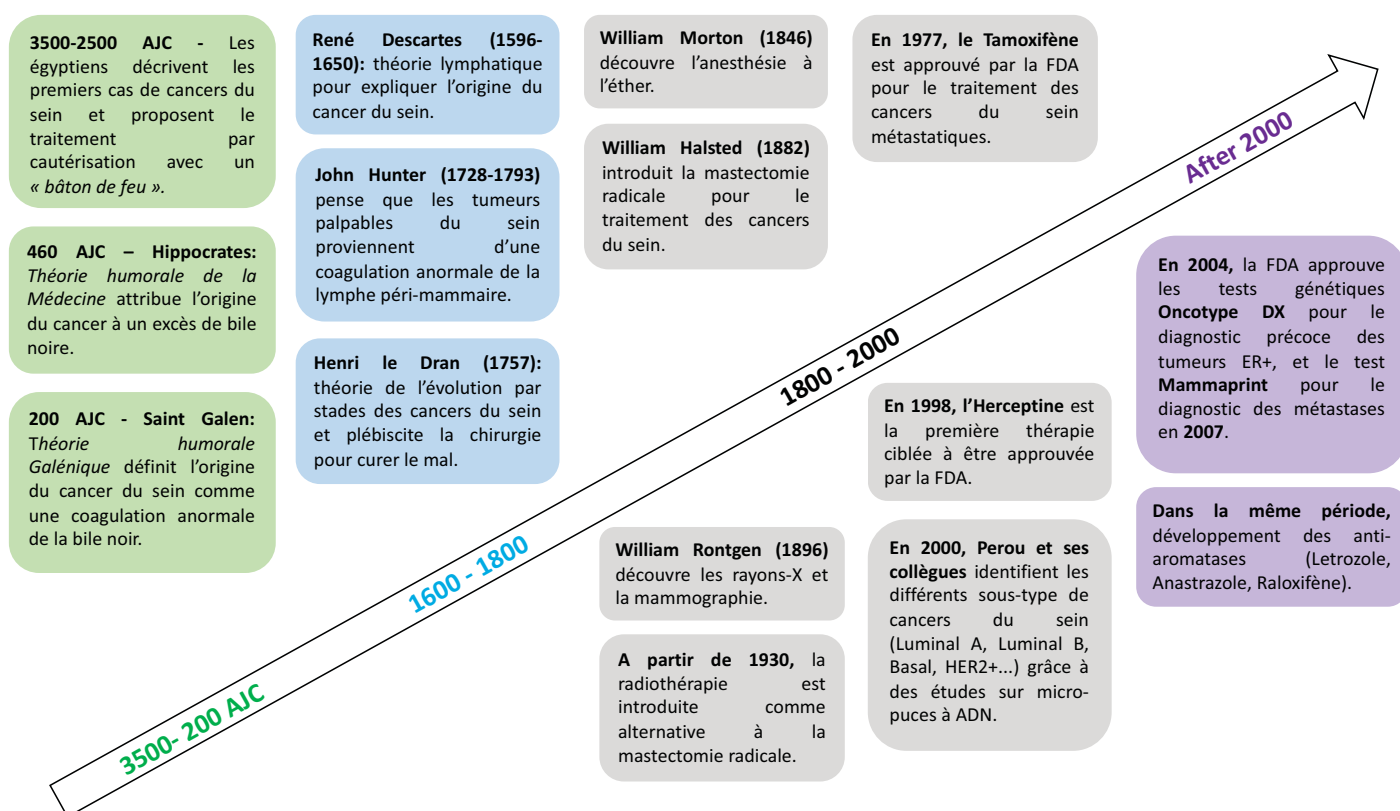


Figure 4 : Historique des cancers du sein depuis 3500 ans avant JC. (adapté de Lukong, 2017)

la progestérone **PR**), sont impliquées dans le développement et la différenciation des cellules du tissu mammaire, pendant la puberté et la grossesse. La progestérone a notamment un rôle dans le développement des acini et l'hypertrophie des cellules sécrétoires (lactocytes) au moment de la lactation. Les oestrogènes quant à eux favorisent le développement des canaux galactophores. Le tissu mammaire est parcouru de vaisseaux sanguins et lymphatiques. Les ganglions lymphatiques sont répartis en différentes zones, notamment au niveau de l'aisselle (ganglions axillaires), dessus et dessous la clavicule (ganglions sus et infra-claviculaires), et autour du sternum (ganglions mammaires internes) (**Figure 2**).

II) Historique des cancers du sein

Les premiers papyrus de l'Égypte ancienne décrivant le cancer du sein datent de 3500 ans. Les données n'étaient alors que très sommaires, et décrivaient le cancer comme étant une « tumeur gonflée des vaisseaux », ou encore une « tumeur proéminente dans la poitrine ». Les médecins préconisaient à l'époque la cautérisation du mal avec un « bâton de feu » (**Figure 3 et 4**) (**Helgason 1987; Brawanski 2012; Lukong 2017**). Par la suite, les théories de la médecine « humorale » décrites par Hippocrate en 460 avant JC., ou encore par Saint Galen en 200 après JC., faisaient état d'une maladie de « l'excès de bile noire » qui contenait des « tumeurs ressemblant à des crabes », d'où l'appellation « karkinos », pour « crabe » en grecque. A cette époque, les traitements proposés pour éradiquer ce « mal » incluaient des infusions d'opium ou d'huile de ricin. Des siècles plus tard, Descartes (XVI^{ème} siècle) et Hunter (XVIII^{ème} siècle) présentaient la théorie « lymphatique », qui réfutait celle de la « bile noire », mais incriminait plutôt la transformation acide du fluide lymphatique péri-mammaire, ou bien encore une anomalie de la coagulation lymphatique dans cette même zone. Ce n'est qu'à la fin du dix-huitième siècle qu'apparurent les premières descriptions modernes du « cancer du sein ». En effet, Henri Le Dran, un grand physicien français de l'époque (1685-1770), présentait le cancer comme une infection initialement locale et qui progressait par étapes, jusqu'à l'infection systémique (développement de métastases). Grâce à ces constatations les premières interventions de résections chirurgicales à proprement parler ont été mises en place, accompagnées de l'élimination de ce que l'on appellera par la suite les « ganglions sentinelles ». L'intérêt de la chirurgie était donc de limiter cette progression maligne en

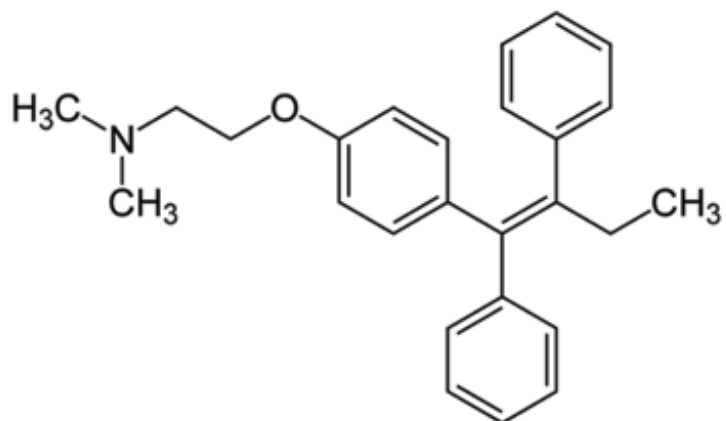


Figure 5 : Structure du Tamoxifène, modulateur sélectif des récepteurs œstrogènes (MSRO), thérapie « antihormones ».

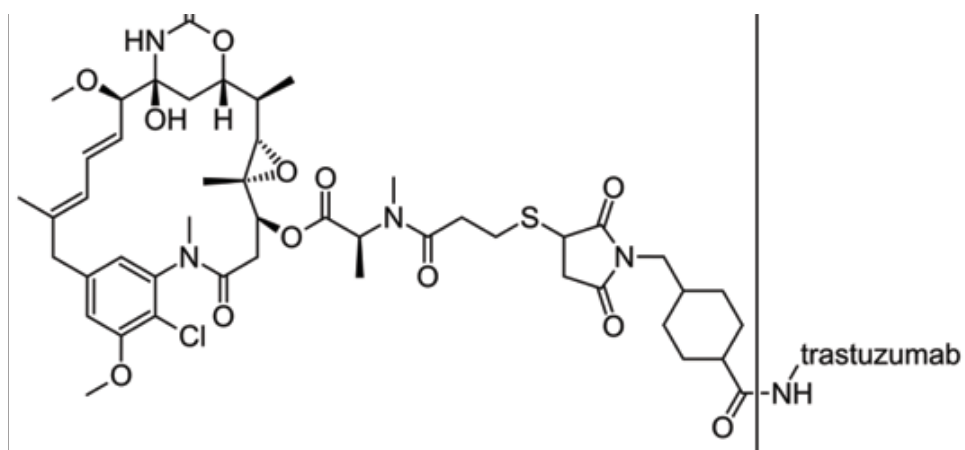


Figure 6 : Structure du Trastuzumab (Herceptine®), anticorps monoclonal recombinant spécifique du récepteur HER2.

éliminant entièrement le tissu lésé avant qu'il ne se propage. Cette pratique sera d'ailleurs maintenue dans les siècles suivants, avec la démocratisation par William Halsted en 1882 de la mastectomie (ou résection extensive du sein). Cette pratique plébiscitée a été rendue plus sûre par la suite, grâce aux méthodes d'anesthésie (**Medical therapy of breast cancer - Rayter, Z. / Mansi, J. - Cambridge University press - Oncologia :: Libreria Cortina Milano s. d.; Lukong 2017**). En outre, une étude clinique réalisée par le Dr Halsted en 1894 démontrait le bénéfice de ces résections mammaires totales sur la survie sans progression. En effet, sur une cohorte de 50 patientes opérées, seulement 3 (6%) avaient récidivé localement après deux ans, contre 56 à 81% de récurrence chez les patientes non opérées. Néanmoins, ces résultats prometteurs ne permettaient pas d'augmenter le taux de survie globale, les femmes étant souvent dépistées à un stade avancé de la maladie (stade métastatique). Par la suite, la découverte des rayons-X (**Röntgen 1896; Lukong 2017**) a permis le développement des premières mammographies, et en 1930, la radiothérapie a été introduite comme alternative à la mastectomie radicale. Le dix-neuvième siècle fut également l'aire de la découverte de l'hormono-dépendance* des cancers du sein. En effet, les tumeurs mammaires détectées chez les patientes ménopausées présentaient des vitesses de prolifération plus faibles que celles des femmes non ménopausées, dont le profil de progression semblait par ailleurs être calqué sur leurs cycles menstruels (**Boyd 1897; Lukong 2017**). Ce n'est qu'au vingtième siècle, dans les années 1980, que les premières thérapies hormonales furent approuvées, avec notamment le développement du Tamoxifène, un Modulateur Sélectif des Récepteurs aux Oestrogènes (**MSRO**)(**Figure 5**) et de l'Herceptine, la première thérapie ciblée à l'encontre du facteur de croissance épidermique humain de type 2 (anti-HER2) (**Figure 6**) (**Tata 2005; Lukong 2017**). Par ailleurs, grâce aux recherches fondamentales et cliniques, les classifications pathologiques et moléculaires des tumeurs mammaires ont vu le jour (**Perou et al. 2000**), tout comme l'existence du caractère héréditaire de certains de sous-types tumoraux, et notamment, l'importance des mutations délétères sur les gènes de réparation de l'ADN (BRCA1 et BRCA2) (**Narod et Foulkes 2004; Ferlay et al. 2015; Lukong 2017**). Ces avancées dans la classification tumorale ont notamment permis le développement de tests génétiques performants. Ces derniers sont basés sur l'analyse de signatures moléculaires prédictives de la réponse des tumeurs aux traitements, tel que *Oncotype DX Breast Cancer Assay* en 2004, (**Kirk 2010; Morris et Carey 2007; Lukong 2017**) et ensuite *MammaPrint* (2007) (**Morris et Carey 2007; Lukong 2017**). Ces tests permettent une approche personnalisée de l'indication

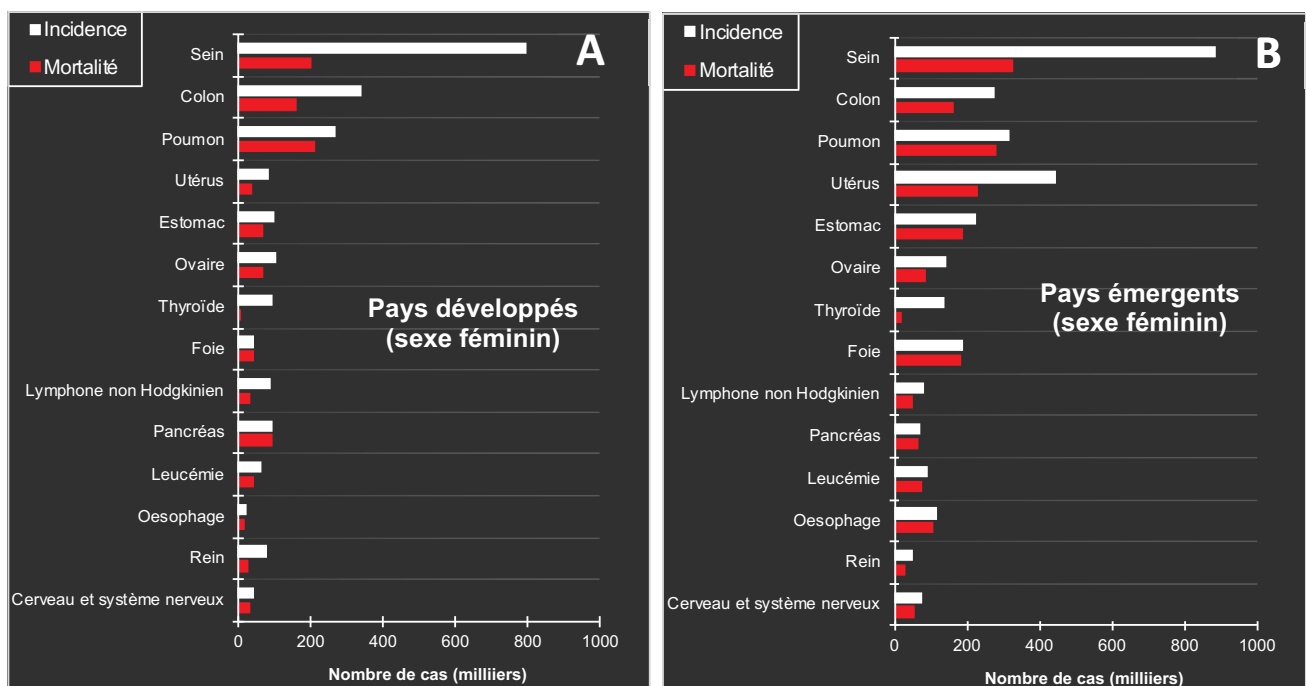


Figure 7 : Incidence et mortalité des cancers chez la femme dans les pays développés (A) et émergents (B) (Ferlay, 2015)

thérapeutique des patientes et permettant ainsi une meilleure prédiction du pronostic des patientes, avec une fenêtre diagnostic d'environ dix ans.

III) Épidémiologie des cancers du sein

Malgré les avancées dans le dépistage et le développement de thérapies, le cancer du sein reste un problème de santé publique majeur. Il se place au second rang mondial de tous les cancers, avec 1,7 millions de cas recensés en 2012. En effet, il représente environ 12% de tous les cancers (**Ferlay et al. 2015**) et il est classé juste derrière les cancers du poumon avec 1,8 millions de cas. Les cancers du sein sont de loin les cancers les plus fréquents chez la femme avec 1,67 millions de femmes diagnostiquées en 2012 (représentant 25% des cancers féminins) (**Figure 7**). Par ailleurs, l'incidence des cancers du sein varie à travers le monde avec un taux de 27 pour 100 000 personnes en Afrique et Asie, contre 96 pour 100 000 en Europe de l'Ouest. Le pronostic des patientes reste tout de même plus favorable dans les pays développés (**Figure 7**), grâce à un meilleur accès au dépistage et aux soins. En termes de mortalité, les cancers du sein se placent au cinquième rang mondial avec un total de 522 000 décès en 2012 (soit 6,4 % de tous les décès). Bien que de nombreux efforts aient été réalisés en termes de prévention, dépistage, de prise en charge et de développement de nouvelles stratégies de traitement (**Lukong 2017**), de grands obstacles restent encore à dépasser. Ces challenges concernent principalement les phénomènes de résistance (**Wu et al. 2014**).

IV) Caractérisation des cancers du sein

Le cancer du sein est une pathologie très complexe et difficile à caractériser. Elle est en effet hétérogène en termes de morphologie, de caractéristiques phénotypiques, de caractéristiques génétiques et de réponse aux traitements (**Haupt, Ro, et Schwartz 2010; Yeo, Turner, et Jones 2014; Advani et Moreno-Aspitia 2014**). La classification des différents types de cancers du sein en rapport avec leur agressivité et leurs caractéristiques biologiques est donc primordiale. Elle permet de pouvoir adapter et optimiser les traitements et ainsi, améliorer le pronostic des patientes.

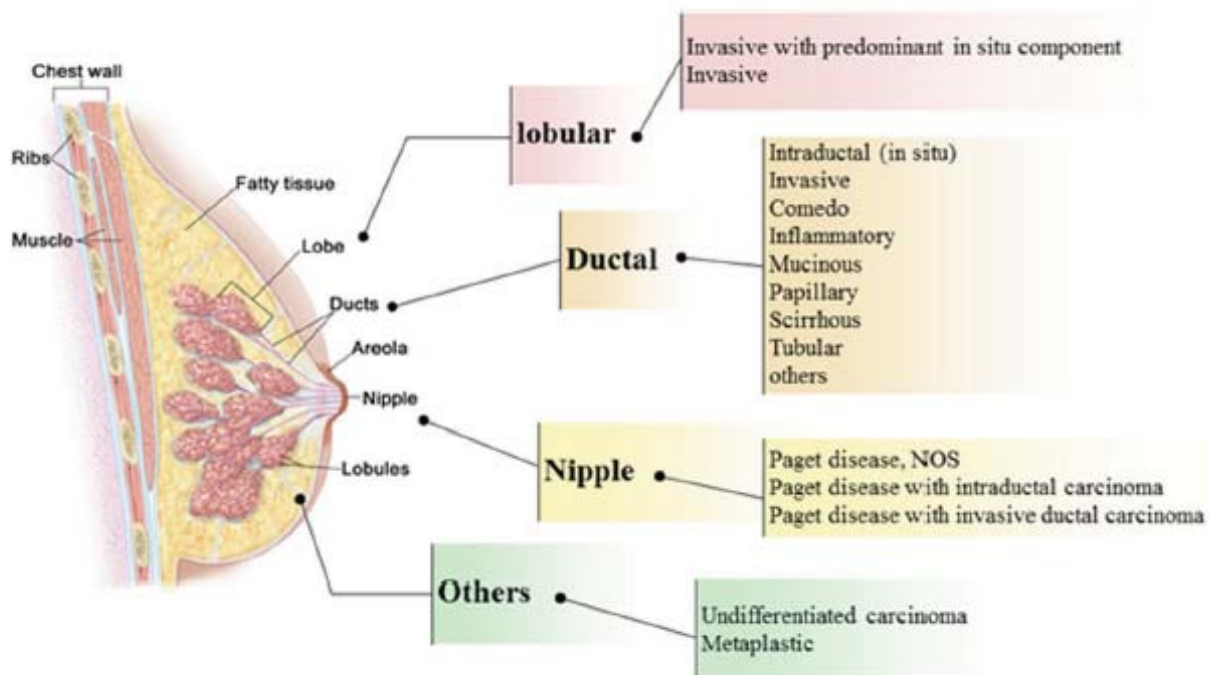


Figure 8: Différents types histologiques des cancers du sein (Pourteimoor, 2016)

Le système TNM distingue le stade clinique pré-thérapeutique noté "cTNM" et le stade anatomopathologique postchirurgical noté "pTNM"

T : Tumeur Primaire T

- **Tx** : la tumeur primitive ne peut pas être évaluée
- **T0** : la tumeur primitive n'est pas palpable
- **T1** : tumeur ≤ 2 cm dans sa plus grande dimension
- **T1mic** : micro-invasion < 1 mm dans sa plus grande dimension
- **T2** : $2 \text{ cm} < \text{tumeur} \leq 5 \text{ cm}$ dans sa plus grande dimension
- **T3** : tumeur $> 5 \text{ cm}$ dans sa plus grande dimension
- **T4** : tumeur, quelle que soit sa taille, avec une extension directe soit à la paroi thoracique (a), soit à la peau (b)

N : Ganglions lymphatiques régionaux pN

- **Nx** : l'envahissement des ganglions lymphatiques régionaux ne peut pas être évalué **N0** : absence d'envahissement ganglionnaire régional histologique et absence d'examen complémentaire à la recherche de cellules tumorales isolées
- **N1mi** : micrométastases $> 0,2 \text{ mm}$ et $\leq 2 \text{ mm}$
- **N1** : envahissement de 1 à 3 ganglions axillaires
- **N2** : envahissement de 4 à 9 ganglions axillaires
- **N3** : envahissement d'au moins 10 ganglions axillaires

M : Métastases à distance (M)

- **Mx** : renseignements insuffisants pour classer les métastases à distance
- **M0** : absence de métastases à distance
- **M1** : présence de métastase(s) à distance

Figure 9 : Classification TNM des cancers du sein, 7e édition 2010 – (canceraquitaine.org)

(a) Classification histologique

La classification des cancers du sein est principalement basée sur des critères morphologiques macroscopiques et histologiques. Ceux-ci permettent de définir le type histologique de la tumeur (par exemple, d'origine canalaire ou lobulaire) ainsi que son degré d'agressivité (différents stades tumoraux*). En effet, les différents stades tumoraux permettent de distinguer (i) les cancers de type localisés non invasifs et sans envahissement ganglionnaire ou cancers *in situ* (stade 0 de la classification), (ii) les formes infiltrantes localement avancées (stade I à III) et (iii) les formes très invasives et métastasées à distance (stade IV). Un certain nombre de formes histologiques ont été décrites par l'OMS, et sont répertoriées dans les classifications de 2003 (Hanby et walker 2004) et 2013 (Sinn et Kreipe 2013; Pourteimoor, Mohammadi-Yeganeh, et Paryan 2016) (Figure 8). Cependant, ce type de classification ne reflétait pas l'hétérogénéité de cette pathologie et surtout, ne permettait pas d'expliquer au sein d'un même type histologique ou d'un même stade tumoral, les différentes réponses aux traitements (Guiu et al. 2012).

Ainsi, en complément de ces classifications, de nouveaux critères basés sur la classification (1) « Taille, Nodules, Métastases (TNM) » (Figure 9) et (2) sur des techniques d'immunohistochimie (IHC) ont permis d'affiner cette stratification (Goldhirsch et al. 2011; Pourteimoor, Mohammadi-Yeganeh, et Paryan 2016).

(1) La classification TNM a été développée afin de définir le degré de sévérité de l'évolution tumorale. Les principaux critères de cette classification sont :

- a. La localisation et la taille de la tumeur primitive (de T0 à T4)
- b. Le degré d'invasion ganglionnaire, par la détermination de l'atteinte ou non des ganglions satellites ou proximaux
- c. La présence et le nombre des métastases (N0 – NX) (Cserni et al. 2018).

d. Le grade histologique de la tumeur ou index histopronostique*, définissant le degré de différenciation des cellules tumorales corrélé au degré d'agressivité (nombre de cellules en mitoses par champ), de **I à IV**.

(2) Les techniques d'IHC permettent quant à elles de définir des biomarqueurs* spécifiques du phénotype tumoral. Leur principe est basé sur :

a. le degré d'expression des récepteurs hormonaux à l'œstrogène (**ER**), à la progestérone (**PR**) et au facteur de croissance épidermique humain de type 2 (**HER2**) (**Penault-Llorca et Viale 2012**). Selon cette classification, 60% des cancers du sein seraient hormonodépendants* (ER+) et 15% ont une amplification du récepteur HER2 (HER2+).

b. Le taux de prolifération, déterminé par l'intensité du marquage de la protéine KI67 (**Penault-Llorca et Viale 2012**).

En effet, en relation avec le degré d'expression de ces récepteurs, il est possible de prédire le degré d'agressivité des tumeurs ainsi que leur réponse aux différents traitements et donc leur pronostic. Ces méthodologies, appliquées actuellement dans le cadre du diagnostic anatomopathologique de routine, ont permis d'améliorer nettement les stratégies de prise en charge des patientes. Néanmoins, le cancer du sein reste une maladie très hétérogène. De ce fait, dans l'objectif d'une meilleure caractérisation, il était nécessaire d'affiner d'avantage sa classification par des études moléculaires.

(b)Classification moléculaire

Depuis une vingtaine d'années, des études transcriptomiques ont permis la mise en place de la classification moléculaire et ainsi, le développement de la thérapie personnalisée (**Duma et al. 2018**). Basées sur la recherche de profils d'expression génique déterminant des signatures moléculaires de tumeurs mammaires, elles ont permis de mettre en évidence

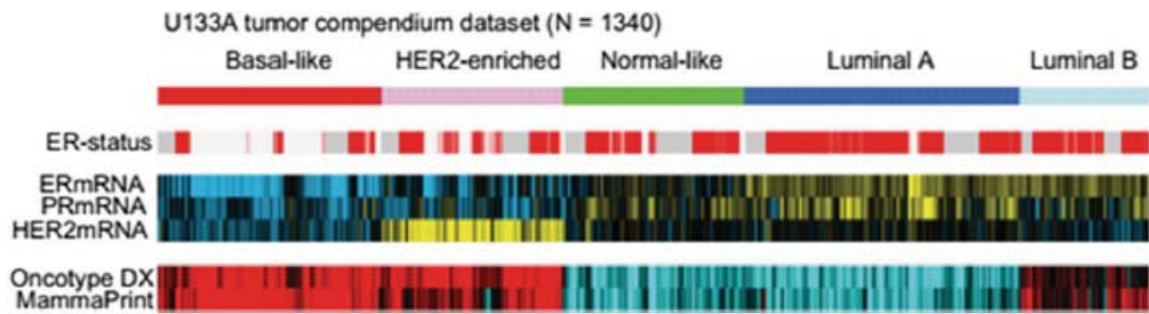


Figure 10 : Différents sous-types moléculaires des tumeurs mammaires (Pourteimoor, 2016)

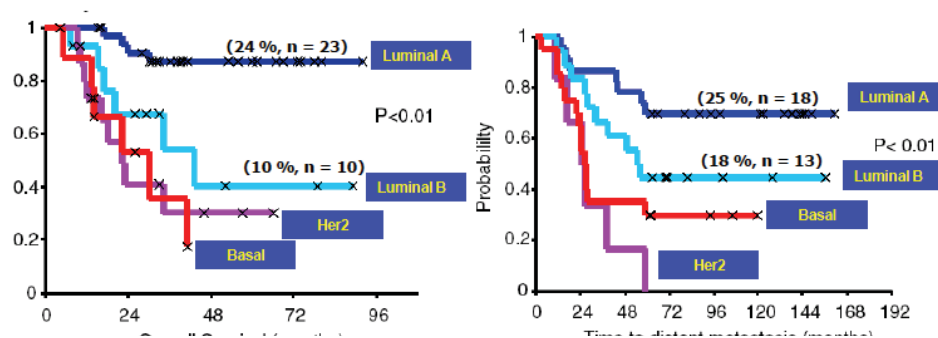
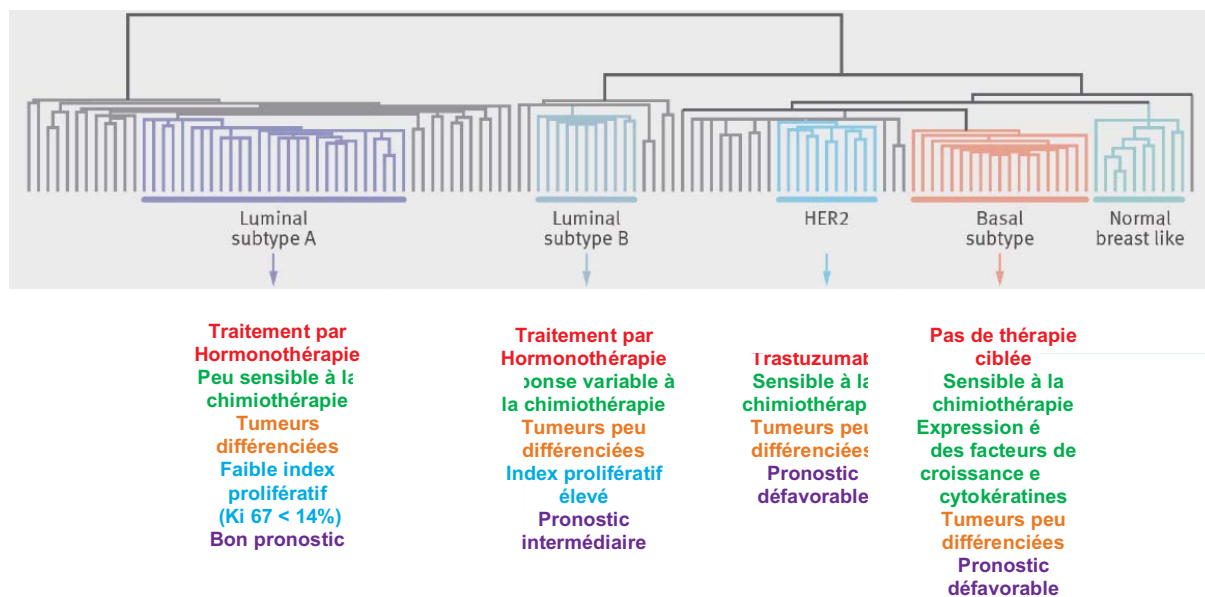
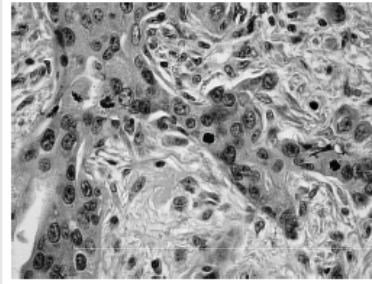
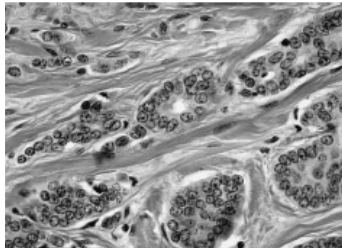


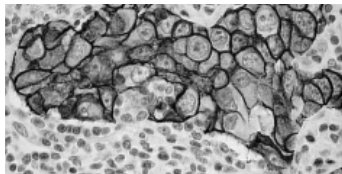
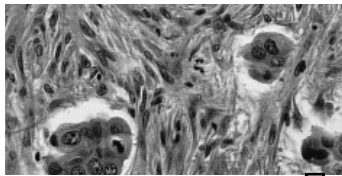
Figure 12 : Valeur pronostique des différents sous-types moléculaires de tumeurs mammaires en termes de survie globale (A) et de durée avant le développement de métastases (B) (Sorlie, 2003)

différents sous-types « moléculaires intrinsèques ». Ces derniers se distinguent principalement par leur niveau d'expression génique des récepteurs aux œstrogènes : récepteurs aux œstrogènes positifs (ER+), et négatifs (ER-), du récepteur à la progestérone (PR+/-) et du récepteur HER2 (Vincent-Salomon et Sigal-Zafrani 2009). Ainsi, la classification moléculaire de Perou en 2000 a mis en évidence cinq sous-types moléculaires intitulés (i) Luminal A, (ii) Luminal B, (iii) ERBB2 (erb-b2 receptor tyrosine kinase 2) amplifié ou « HER2 surexprimé », (iv) *basal-like* et (v) *Normal Breast-Like*. Ce dernier sous-type « *Normal Breast-Like* » s'avère être un artéfact de prélèvement ne présentant pas de traduction réelle en termes de pathologie et n'est plus pris en compte dans cette classification (Perou et al. 2000; Vincent-Salomon et Sigal-Zafrani 2009) (Figure 10). Ensuite, un autre sous-type moléculaire « Claudin-low » a été identifié (Prat et al. 2010).

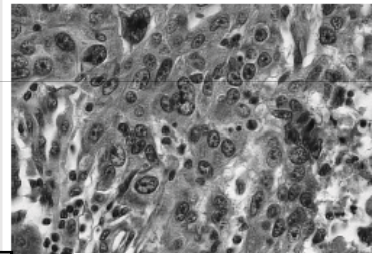
1. Les tumeurs **luminales A** représentent 60% des cancers du sein. De relativement bon pronostic, elles se caractérisent par des cancers de bas grade (I et II) exprimant fortement les récepteurs aux oestrogènes (ER++) et présentant des index de prolifération faibles (Sørli et al. 2001; Perou et al. 2000) (Figures 11, 12 et 13).
2. Les tumeurs de type **Luminal B** représentent quant à elles 20 à 25% des tumeurs mammaires et expriment plus faiblement les récepteurs aux oestrogènes (ER+/-), mais très fortement les gènes de prolifération. Ces tumeurs sont en général plus agressives (de grade II ou III) et très proliférantes (Sørli et al. 2001; Perou et al. 2000) (Figures 11, 12 et 13).
3. Le troisième sous-type de tumeurs mammaires « **HER2** » (15% des cas) se caractérise par une forte amplification du gène ERBB2, et peut être divisé en deux sous-classes : celle exprimant fortement les ER (ER+), et celle ne les exprimant pas (ER-). Selon leur statut d'expression du récepteur ER, elles ont des pronostics variables (Sørli et al. 2001; Perou et al. 2000) (Figures 11, 12 et 13).
4. Finalement, le sous-type de tumeurs mammaires **basal-like** (15% des cas), se caractérise par une absence d'expression de ER (ER-) et de la progestérone (PR-), ainsi



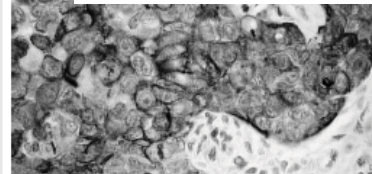
b. Exemple de carcinome de type luminal B (coloration HES).



Marquage fort du récepteur HER2
HC (type HER2+)



Exemple



Marquage fort des cytokératines
5 et 6 en IHC (type basal-like)

immunohistochimie (IHC) – Salomon, 2009)

Sous-type selon le profil d'expression génique (GEP)	Classification Immunohistochimique (IHC) (Saint Gallen*)			
Luminal A		-	< 14%	73 à 100%
Luminal B				73 à 100 %
	+	-	> 14%	
	Phénotype "HER2 positif"			
	+		Indifférent	
HER2 Surexprimé				4:
	-	Sur-exprimé ou amplifié	Indifférent	
Basal-like				80%

ER: récepteur aux oestrogènes. PR: récepteur à la progestérone. HER2: récepteur au facteur de croissance épidermique humain 2. Ki-67:antigène Ki-67, marqueur de prolifération cellulaire (Giu S. et al., 2012).

qu'une absence d'amplification de ERBB2 (**Sørli et al. 2001; Perou et al. 2000**). Par ailleurs, les tumeurs *basal-like* expriment fortement les cytokératines 5 et 17, marqueurs des cellules de la membrane basale, ainsi que l'intégrine b4 et la laminine 1 (**Anders et al. 2016**). D'un point de vue clinique, les tumeurs *basal-like* sont souvent de type canalaire de haut grade (III), agressives, peu différenciées et présentant des index mitotiques élevés. Par ailleurs, ces tumeurs présentent des marges refoulantes contenant des plages de nécrose centrale ainsi que des infiltrats lymphocytaires périphériques (**Figures 11, 12 et 13**).

5. Un cinquième sous-type moléculaire intitulé ***Claudin-Low*** et apparenté aux cellules souches mammaires a par la suite été décrit (**Prat et al. 2010**). Celui-ci, plus rare, se caractérise par (i) une absence d'expression des protéines de jonctions serrées telles que les claudines 3, 4 et 7 et l'E-cadhérine, (ii) une faible expression des marqueurs de luminaux, et (iii) une forte expression des gènes de la réponse immunitaire et des gènes caractérisant les cellules-souches. La majorité des tumeurs *Claudin-low* n'expriment pas les récepteurs hormonaux (ER-, PR-), et sont de mauvais pronostic (**Prat et al. 2010, 2015**). En termes de réponse clinique, ce sous-type présente une sensibilité aux thérapeutiques intermédiaire entre les sous-types luminaux et *basal-like* (**Turashvili et Brogi 2017**).

La découverte de ces différents sous-types moléculaires a permis d'affiner la classification immunohistochimique et de prendre en compte ces différentes sous-classes tumorales (**Figure 14**). Le recouvrement entre ces deux classifications n'est pas complet, mais a permis de mettre en évidence des sous-types phénotypiques d'intérêt clinique et notamment, le sous-type de tumeurs *basal-like*, contenant le phénotype « Triple-Négatif », majoritaire et très agressif (**Guiu et al. 2012; Anders et al. 2016; Goldhirsch et al. 2011**).

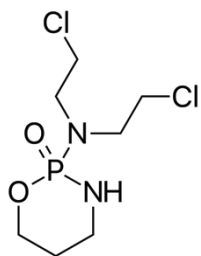


Figure 15 : Structure du Cyclophosphamide

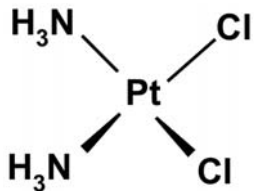


Figure 16 : Structure du Cisplatine

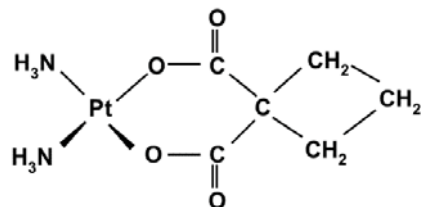


Figure 17 : Structure du Carboplatine

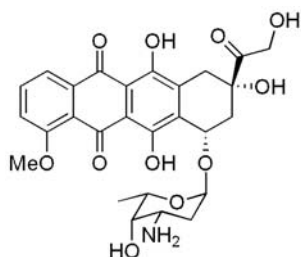


Figure 18 : Structure de la Doxorubicine

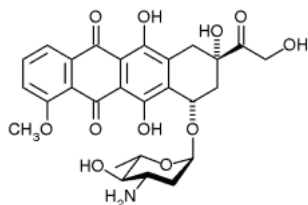


Figure 19 : Structure de l'épirubicine

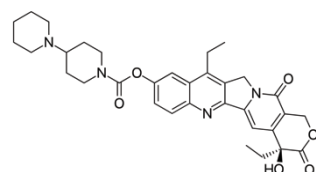


Figure 20 : Structure de l'Irinotécan

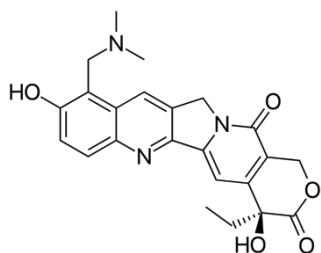


Figure 21 : Structure du Topotécan

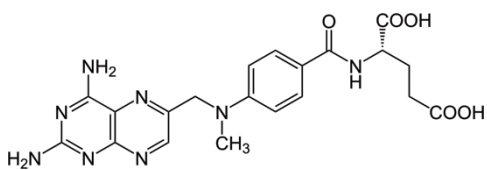


Figure 22 : Structure du Methotrexate

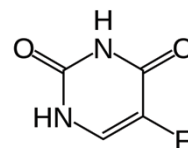


Figure 23 : Structure du 5-Fluorouracile

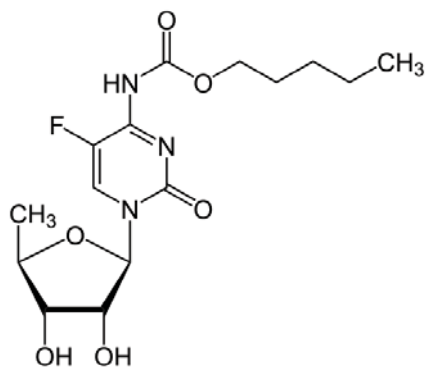


Figure 24 : Structure de la Capécitabine

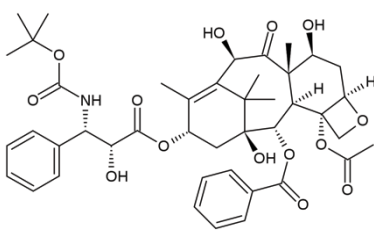


Figure 25 : Structure du docétaxel

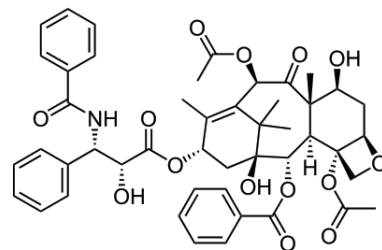


Figure 26 : Structure du Paclitaxel

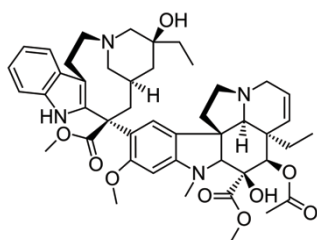


Figure 27 : Structure de la Vinblastine

V) La prise en charge des cancers du sein

Compte tenu de la grande diversité des tumeurs mammaires, différentes stratégies de prise en charge thérapeutique peuvent être envisagées telles que la chirurgie, la radiothérapie*, la chimiothérapie*, l'hormonothérapie*, et les thérapies ciblées* (**Yeo, Turner, et Jones 2014; PDQ Adult Treatment Editorial Board 2002**). Les cliniciens prennent connaissance du tableau clinique de la patiente, qui comprend (i) la détermination du statut ménopausal et de l'antériorité des traitements délivrés, (ii) l'analyse des données de mammographie, d'échographie, (iii) d'imagerie par résonance magnétique (IRM) et bien sûr (iv) l'analyse anatomopathologique de la tumeur. Grâce à ces examens, le grade, le type histologique et l'expression des biomarqueurs d'intérêt clinique sont identifiés (**Yeo, Turner, et Jones 2014**). Sur la base de ces paramètres, l'équipe médicale pluridisciplinaire fait le choix des approches thérapeutiques les plus adaptées. Cette stratégie dépendrait dans un premier temps du stade tumoral, c'est-à-dire le degré d'extension de la tumeur, qui peut être localisé (stade 1 de tumeur *in situ*) jusqu'à avancé (stade 4 de tumeur métastatique).

(a) La chimiothérapie

La chimiothérapie correspond à un traitement par un ensemble de molécules ayant des activités cytotoxiques* et/ou cytostatiques*. Parmi les principales classes de molécules, on retrouve les agents alkylants* (cyclophosphamide, cisplatine, carboplatine) (**Figures 15, 16 et 17**), les agents intercalants* (doxorubicine, épirubicine) (**Figures 18 et 19**), les inhibiteurs de topoisomérases* (Irinotécan, Topotécan) (**Figures 20 et 21**), les antimétabolites* (methotrexate, 5-fluorouracile et la capécitabine) (**Figures 22, 23, 24**), et les agents antimitotiques* (docétaxel, paclitaxel, vinblastine) (**Figures 25, 26 et 27**). Afin d'augmenter l'efficacité des thérapies, des co-administrations sont également indiquées, telles que les protocoles **FEC** combinant le 5-Fluorouracile, l'épirubicine, et le cyclophosphamide, ou **FAC** combinant le 5-Fluorouracile, la doxorubicine et le cyclophosphamide) (**PDQ Adult Treatment Editorial Board 2002**).

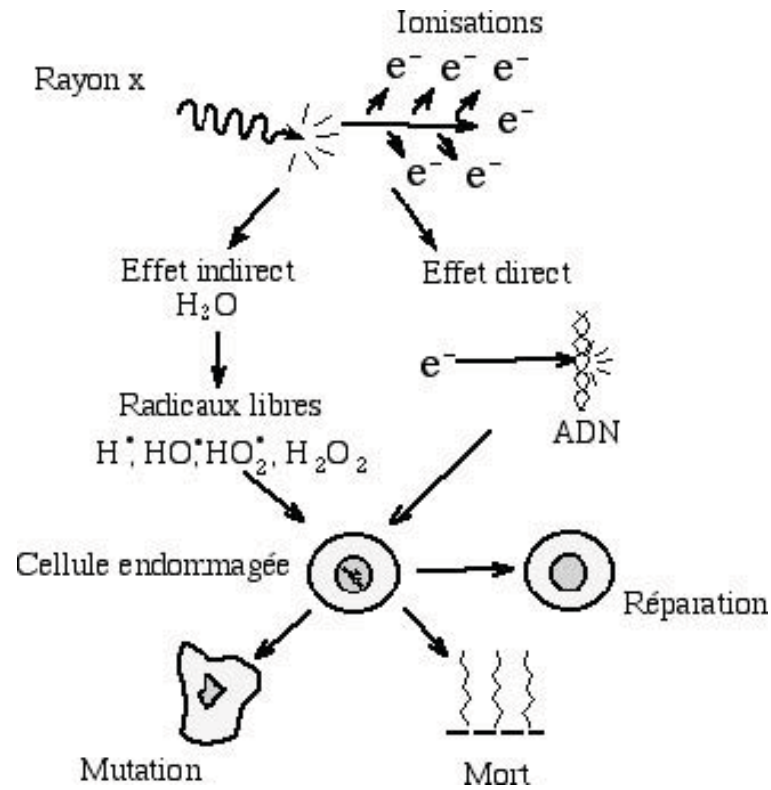


Figure 28 : Effets cellulaires directs et indirects de la radiothérapie
 (http://radioactivite4.free.fr/radioactivite.Les_effets_biologiques)

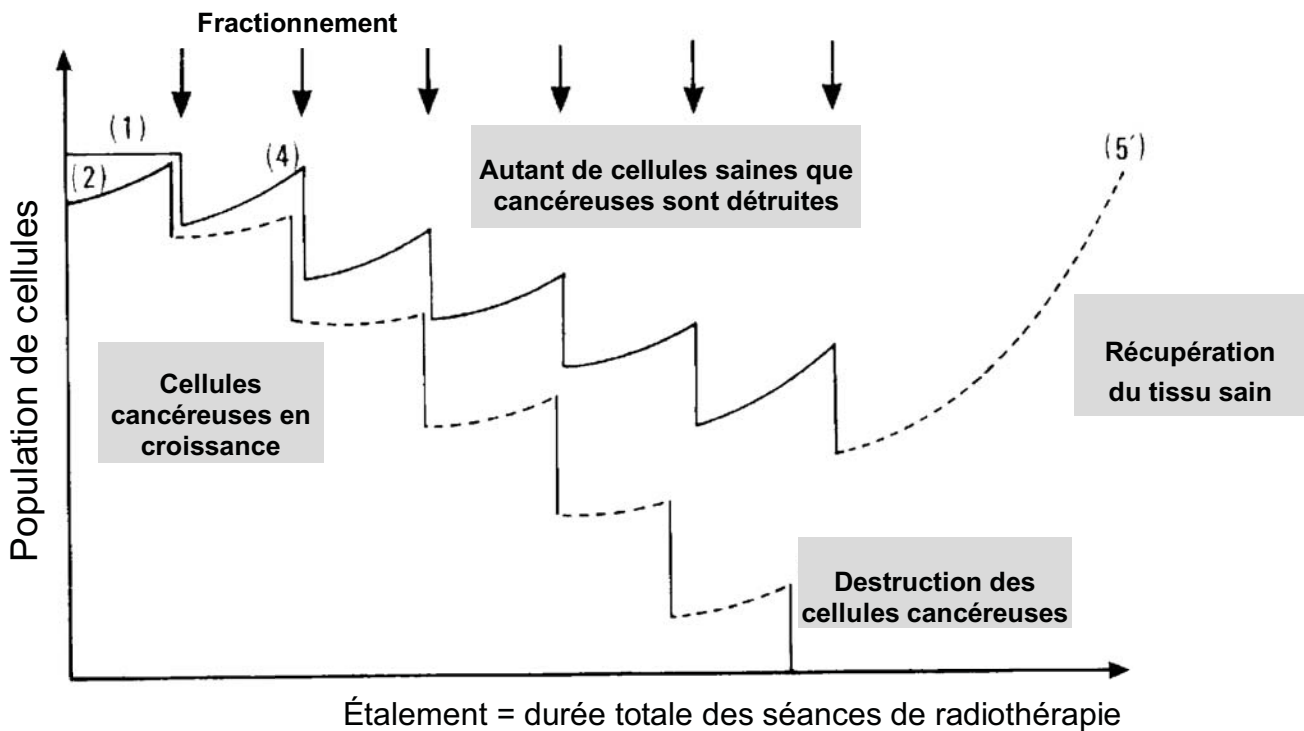


Figure 29 : Intérêt du fractionnement et de l'étalement des séances de radiothérapie afin de détruire spécifiquement les cellules cancéreuses et préserver les tissus sains (Viau, 2016).

Pour le traitement des cancers du sein, les principaux protocoles sont basés sur des combinaisons **FEC** ou **FAC** en association avec des agents antimitotiques tels que le paclitaxel ou le docétaxel. La dose, le nombre de cures et leur durée dépendent du statut de la tumeur et du niveau de tolérance des patientes.

(b) La radiothérapie

La radiothérapie implique l'utilisation de différents types de rayonnements ionisants afin d'induire des lésions létales de l'ADN. Ces rayonnements peuvent être de types (i) électromagnétiques (photons), (ii) particuliers chargés (électrons, protons, rayonnement alpha, ions lourds) ou (iii) non chargés (neutrons). Ces différentes sources de rayonnements présentent des rendements en profondeur variables, et sont choisis selon le type de tumeur à traiter. La radiothérapie induit des lésions de l'ADN par ionisation directe ou par ionisation des molécules d'eau environnantes, formant des radicaux libres hautement réactifs (**Figure 28**). Cette étape indirecte est aussi appelée radiolyse de l'eau. Quel que soit le mécanisme, on estime qu'un Gray (Gy) de radiothérapie (unité définissant la dose absorbée par l'eau ou un tissu) génère plus de 1000 cassures simple-brin (**CSBs**) et environ 40 cassures double brin (**CDBs**) par cellule irradiée (**Dornfeld et al. 2011; Linberg et al. 1998**). En outre, l'efficacité biologique de la radiothérapie dépend des critères suivants :

1. La capacité de réparation de dommages des tissus sains vs les tissus tumoraux. L'objectif des programmes de radiothérapie est d'étaler correctement les séances afin de laisser le temps au tissu sain de se régénérer, mais d'empêcher la réparation des lésions tumorales.
2. La distribution des cellules au sein du cycle cellulaire. En effet, les cellules les plus sensibles à la radiothérapie sont celles en phase **G2** ou **M**, lorsque les mécanismes de réparation de l'ADN sont les moins actifs.

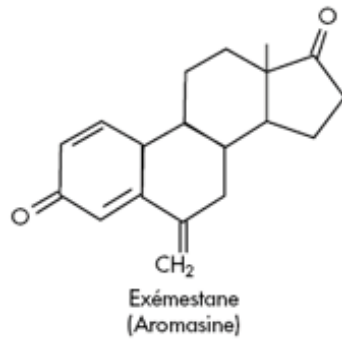
3. L'oxygénation tissulaire. En effet, les tumeurs possèdent une proportion de cellules hypoxiques (15% du volume tumoral). Or, ces cellules sont très peu sensibles aux rayonnements ionisants. Ainsi, très rapidement après les séances de radiothérapie, les cellules normoxiques sont préférentiellement éliminées, tandis que les cellules hypoxiques peuvent persister. Il est alors nécessaire d'espacer suffisamment les séances afin de permettre aux tumeurs de se ré-oxygéner (**Dornfeld et al. 2011; Linberg et al. 1998**).
4. La radiosensibilité intrinsèque des cellules, notamment définie par le degré d'efficacité des systèmes de réparation de l'ADN. En effet, un des facteurs de sensibilité à la radiothérapie est la présence d'un système de réparation des cassures déficient chez les cellules tumorales (**Kötter et al. 2014; Lesueur et al. 2017**).

Un protocole de radiothérapie s'établit donc en déterminant le type de **fractionnement** et l'**étalement des séances**. Le type de **fractionnement** définit à la fois le nombre de fractions administrées, et la dose par fraction (par exemple, 5 fractions de 2 Gy par semaine). L'**étalement** correspond à la durée totale entre la première et la dernière séance de traitement. Ces deux paramètres ont pour objectif de créer un différentiel maximal entre le tissu cancéreux et le tissu sain afin de spécifiquement éliminer les cellules cancéreuses (**Figure 29**)(**Viau et al. 2016**). Ils permettent également de limiter les complications précoces sur des tissus sains à prolifération rapide ou tardives, et sur des tissus sains à prolifération lente. Par ailleurs, un mauvais étalement des séances ou un non-respect du fractionnement peut induire un échappement des cellules tumorales, et donc un échec thérapeutique.

(c) Traitement des cancers du sein localisés

Dans le cas de cancers localisés, et quel que soit le sous-type tumoral, le traitement repose en premier lieu sur une chirurgie conservatrice (tumorectomie) ou non conservatrice (mastectomie), suivie le plus souvent de séances de radiothérapie (**Bhoo-Pathy et al. 2015**). La chirurgie vise à éliminer la tumeur, et parfois les ganglions axillaires pouvant être le siège de cellules métastatiques. Ces stratégies permettent de limiter fortement le risque de

Inhibiteurs d'aromatase de type I (stéroïdiens)



Inhibiteurs d'aromatase de type II (non stéroïdiens)

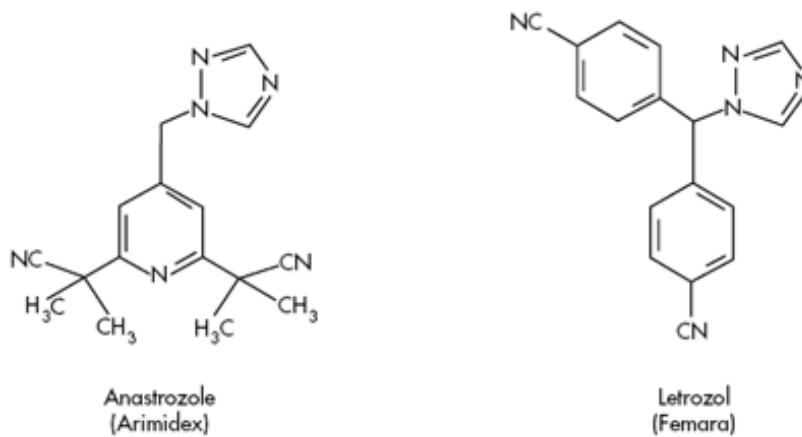


Figure 30 : Structure des anti-aromatases

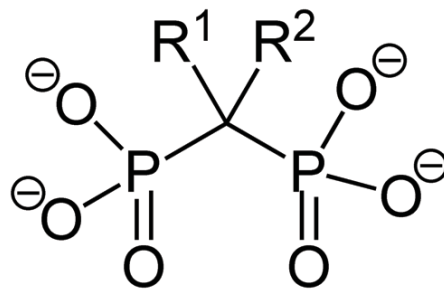


Figure 31 : Structure des biphosphonates - Les groupements R déterminent les propriétés chimiques des molécules, les distinguant entre les différentes classes de biphosphonates. Leur structure chimique leur confère une haute affinité pour le calcium hydroxyapatite, permettant une fixation osseuse rapide et spécifique.

récidive, et d'augmenter la survie globale. Ensuite, un suivi régulier des patientes est réalisé dans les années suivant la prise en charge (**Moo et al. 2018; Yeo, Turner, et Jones 2014**).

(d) Traitement des cancers localement avancés

Lorsque le cancer est localement avancé, c'est-à-dire infiltrant mais non métastatique, le traitement débutera également par de la chirurgie (conservatrice ou non). Ensuite, des séances de radiothérapie seront réalisées sur le lit tumoral et sur les aires ganglionnaires afin de limiter le développement des métastases et d'éliminer les éventuels foyers cancéreux. Dans certains cas, lorsque la tumeur est inflammatoire ou trop volumineuse pour être réséquée d'emblée, des séances de radiothérapie ou des traitements néoadjuvants* de chimiothérapie ou d'hormonothérapie sont proposés, avant la chirurgie, afin de réduire le volume tumoral (**Orecchia 2018**). Ensuite, selon le risque de récurrence de la patiente, des thérapies adjuvantes spécifiques sont prescrites. Ainsi, pour des tumeurs ER+ localement avancées, la thérapie adjuvante proposée est le Tamoxifène® (**Panasci et al. 1996**) (**Figure 5**), un modulateur sélectif du récepteur aux oestrogènes. Selon une étude réalisée en 2014, le traitement par Tamoxifène permet de réduire le risque de rechute de 41%, et le risque de mortalité de 31% (**Yeo, Turner, et Jones 2014; Advani et Moreno-Aspitia 2014**). Ce traitement est le standard pour traiter les patientes préménopausées, et est administré pendant au moins cinq ans (et peut aller jusqu'à 10 ans dans certains cas). En revanche, pour les patientes ménopausées présentant des tumeurs ER+, les anti-aromatases sont plutôt prescrites (**Figure 30**), ayant démontré un effet supérieur au Tamoxifène. Par ailleurs, pour les cancers du sein HER2+ présentant un pronostic intrinsèquement moins bon que les autres sous-types de cancers du sein (**Yeo, Turner, et Jones 2014**), la thérapie adjuvante* administrée est le Trastuzumab, un anticorps monoclonal bloquant le domaine extracellulaire du récepteur HER2 (**Figure 6**) (**Tong et al. 2018; Escrivá-de-Romaní et al. 2018**). Administré toutes les trois semaines pendant un an, il augmente la survie globale et la survie sans rechute, et engendrerait peu d'effets secondaires (**Yeo, Turner, et Jones 2014**). En revanche, pour les cancers du sein avancés non hormonodépendants (ER-) ou résistants aux hormones et ne surexprimant pas HER2 (HER2-), la chimiothérapie est la seule option. Les agents de chimiothérapie proposés sont à base d'agents antimétaboliques (ex : paclitaxel), d'agents

intercalants (ex : doxorubicine), d'antimétabolites (ex : 5-fluorouracile), ou d'agents alkylants (ex : cisplatine, cyclophosphamide). Selon la réponse tumorale, ces traitements peuvent être administrés en première, deuxième ou troisième ligne d'intention. Le choix du type de chimiothérapie et de leur combinaison dépend des traitements antérieurs et du potentiel d'acceptation de la toxicité associée à ces traitements, notamment pour celle liée au paclitaxel (Taxol®) ou à la capécitabine (Xeloda®). En revanche, la durée optimale du traitement de chimiothérapie n'a pas obtenu de consensus **(Yeo, Turner, et Jones 2014)**.

(e) Traitement des cancers avancés métastatiques

Lorsque le cancer du sein est avancé et métastatique, le traitement préférentiel est la chimiothérapie ou l'hormonothérapie néoadjuvante. Le rôle de cette thérapie systémique est de contrôler le développement de la maladie (l'invasion), de diminuer les symptômes, et d'augmenter la survie sans progression. Par ailleurs, la médiane de survie des cancers avancés dépend souvent du sous-type tumoral. En effet, en cas de maladie métastatique, la médiane de survie est estimée à 2,2 ans pour les tumeurs de sous-type luminal A, contre seulement 6 mois pour les tumeurs *basal-like* **(Yeo, Turner, et Jones 2014)**. Pour les cancers métastatiques avancés surexprimant HER2 (HER2+) ou ER+, le traitement par Tamoxifène ou Trastuzumab est recommandé comme première ligne de traitement, avant la chimiothérapie. Ensuite, le choix du traitement dépendra des traitements antérieurs et du statut ménopausal de la patiente. La chirurgie peut être réalisée lorsque l'état de la patiente le permet, et lorsque les tumeurs ou lésions métastatiques sont localisées dans des zones empêchant le bon fonctionnement des organes.

De plus, la gestion des douleurs liées aux métastases doit également faire l'objet d'une prise en charge particulière. En effet, 60 à 80% des patientes présentent des métastases osseuses, incluant des douleurs, des risques de fractures, et de compression vertébrale. Ces effets sont réduits par la prise de bisphosphonates **(Figure 31)**, inhibant la résorption ostéoclastique. Un autre site fréquent de métastases est le cerveau (notamment chez les patientes porteuses de tumeurs TN), impliquant des problèmes de pénétration des

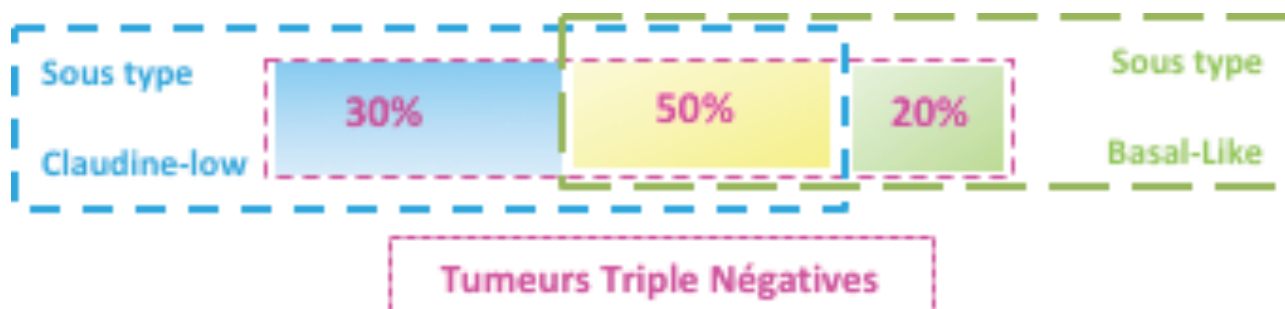


Figure 32 : Répartition des tumeurs Triple-Négatives entre le sous-type moléculaire « basal-like » et « claudine-low ». 50% des tumeurs TN ont un profil moléculaire mixte entre ces deux sous-types.

traitements à travers la barrière hémato-encéphalique. Ainsi, dans le cas de métastases multiples, une radiothérapie peut être proposée (**Brown et al. 2017; El Shafie et al. 2018**). En revanche, pour les métastases isolées, la chirurgie stéréotaxique* est préférentiellement réalisée (**Yeo, Turner, et Jones 2014**). Cependant, la maladie métastatique reste incurable pour une majorité de patientes (seulement 20% de survie globale à 5 ans), bien que leur qualité de vie puisse être maintenue pendant plusieurs années (**Khodari et al. 2013**).

B) Les cancers du sein Triple-Négatifs

I) Caractéristiques

Les tumeurs mammaires « Triple-Négatives » (TN) représentent 15 à 20% des cancers du sein et sont très agressives. Elles sont des carcinomes infiltrants de type canalaire et de haut grade (Badve et al. 2011). Les tumeurs TN se caractérisent par une absence d'expression phénotypique des récepteurs hormonaux ER, PR, ainsi qu'une absence de surexpression du récepteur HER2 (ER-, PR-, HER2-). Elles expriment fortement les cytokératines 5, 6 et 17, le récepteur à l'EGF (EGFR) et le proto-oncogène* c-kit. Par ailleurs, ces tumeurs contiennent fréquemment des mutations sur le gène p53 (dans presque 100% des cas), sur PTEN, et sur les gènes de réparation de l'ADN, notamment le BRCA1 ou BRCA2 (**Anders et al. 2016; Anders et Carey 2009**). De plus, leur incidence est souvent associée à une prédisposition héréditaire par des mutations germinales sur les gènes BRCA1 et/ou BRCA2. Ces caractéristiques les classent majoritairement dans le sous-type moléculaire des tumeurs mammaires *basal-like* (70 à 80% de concordance entre la classification moléculaire et l'IHC). Par ailleurs, les tumeurs TN présentent un faible taux d'expression de l'E-cadhérine et des gènes impliqués dans l'adhésion intercellulaire (**Prat et Perou 2011**), ce qui permet de les classer également dans le sous-type moléculaire des *Claudin-low* (dans environ 30% des cas) (**Anders et al. 2016; Prat et Perou 2011; Banda et al. 2014**) (**Figure 32**). Ainsi, ces tumeurs sont très hétérogènes en termes de caractéristiques moléculaires, mais également en termes d'épidémiologie et de réponses aux traitements. Par ailleurs, elles touchent majoritairement des femmes jeunes d'origine afro-américaine âgées de 30 à 50 ans. A titre de comparaison, les patientes atteintes d'autres sous-types tumoraux présentent un âge médian au diagnostic de 60 ans. De plus, les

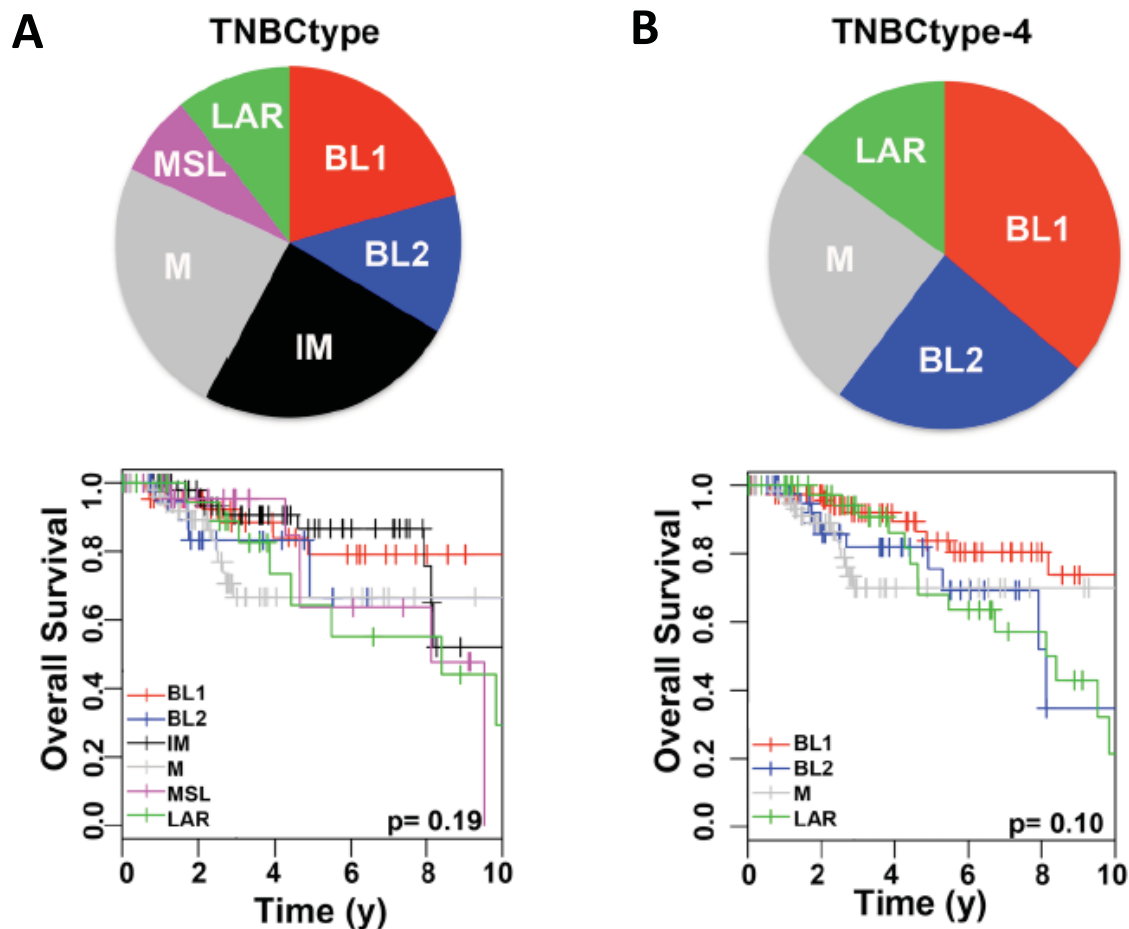


Figure 33 : Classification moléculaire et survie globale (figures de Kaplan Meier) des tumeurs mammaires Triple-Négatives comprenant les six sous-types initiaux : basal-like1 et basal-like2 (BL1 et BL2), mesenchymal (M), luminal androgen receptor (LAR), immunomodulatory (IM) et mesenchymal stem-like (MSL) (A) et la mise à jour de cette classification avec seulement quatre sous-types (B), les sous-types mesenchymal (M) et immunomodulatory (IM) étant considérés comme des descripteurs de l'hétérogénéité cellulaire au sein des tumeurs TN. (Lehmann 2016).

métastases des tumeurs TN sont majoritairement cérébrales (50% des métastases des tumeurs TN), et pulmonaires (Sulaiman et Wang 2017; Anders et al. 2016). Ce qui les différencie des autres sous-types tumoraux qui le plus souvent développent des métastases au niveau osseux (Lehmann et al. 2016). De par leurs caractéristiques, ces tumeurs sont généralement très agressives et de pronostic défavorable.

II) Classification moléculaire

La forte hétérogénéité des tumeurs TN a mené les chercheurs à définir différents sous-types moléculaires. Ainsi, la classification des tumeurs TN de Lehmann (Lehmann et al. 2011) décrit les six sous-types moléculaires suivants : *basal-like1* et *basal-like2* (BL1 et BL2), *mesenchymal* (M), *luminal androgen receptor* (LAR), *immunomodulatory* (IM) et *mesenchymal stem-like* (MSL) (Figure 33). Chaque sous-type présente des caractéristiques moléculaires uniques, et une réponse différentielle à la chimiothérapie de référence. En effet, le sous-type BL1 se caractérise par une forte expression des gènes de réparation de l'ADN et du cycle cellulaire, tandis que le BL2 est enrichi en marqueurs myoépithéliaux et en facteurs de croissance. Les deux sous-types M et MSL présentent une forte expression des gènes impliqués dans la transition épithélio-mésenchymateuse* (ou TEM), avec, spécifiquement pour le sous-type MSL, une faible expression des gènes de la prolifération. Le sous-type IM quant à lui présente une forte expression des gènes des voies de l'immunité. Finalement, le sous-type LAR est caractérisé par l'expression des gènes « luminaux », et une augmentation de l'expression des récepteurs aux androgènes.

Par la suite, ces six sous-types ont été regroupés en quatre principaux qui sont les sous-types *basal-like 1* (BL1), *basal-like 2* (BL2), *mesenchymal* (M), et *luminal androgen receptor* (LAR) (Lehmann et al. 2016). En effet, les deux sous-types *mesenchymal* (M) et *immunomodulatory* (IM) se sont avérés être des descripteurs de l'hétérogénéité cellulaire au sein des tumeurs TN, décrivant notamment une forte proportion de stroma dans la tumeur pour le descripteur « M » ou d'infiltrats lymphocytaires (20% des cas) pour le descripteur « IM » (Figure 33). Par ailleurs, ces quatre nouveaux sous-types présentent des

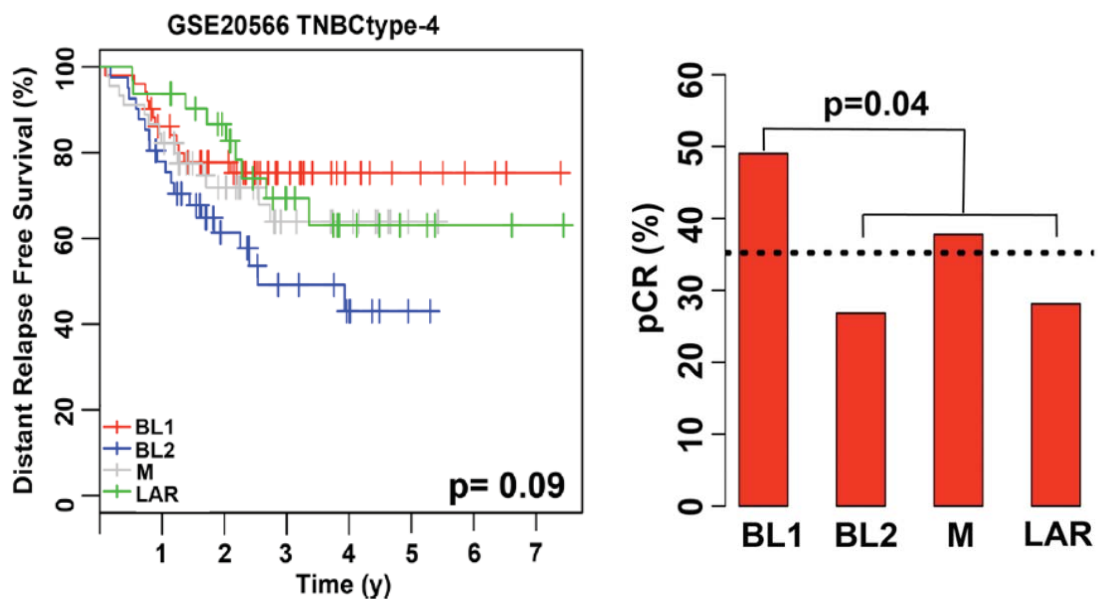


Figure 34 : Survie globale des différents sous-types de tumeurs TN suite au traitement néoadjuvant par anthracyclines et taxanes (Lehmann 2016)

caractéristiques très spécifiques en termes d'âge des patientes au diagnostic, de grade, de type histologique et de profil de progression (progression locale ou à distance). Ils diffèrent également en termes de réponse à la chimiothérapie par anthracyclines suivie d'un traitement par des taxanes, avec 50% de réponse complète pour le sous-type BL1 et seulement 25% pour le sous-type BL2 (**Figure 34**). Ces résultats suggèrent donc une réponse de nature « sous-type dépendante » des tumeurs TN à la chimiothérapie néoadjuvante. De plus, ils soulignent l'importance de l'hétérogénéité moléculaire des tumeurs TN dans la prise en charge des patientes.

III) Prédispositions génétiques

Les tumeurs TN se caractérisent également par une forte instabilité génétique. Elles présentent notamment des déficiences dans leurs systèmes de réparation des CDBs de l'ADN par recombinaison homologue (**HR**). Cette déficience a par ailleurs été décrite comme un des moteurs du développement tumoral. En effet, les protéines impliquées dans les mécanismes de réparation de l'ADN *via* le système **HR** sont BRCA1 et BRCA2 (codées par les gènes du même nom). Elles ont été identifiées dans les années 1990 comme étant des suppresseurs de tumeurs* (**Dhillon et al. 2016**). Ces protéines sont considérées comme des garants du maintien de l'intégrité génomique. En effet, outre leur implication dans le système **HR**, elles sont également responsables de la différenciation des cellules mammaires et ovariennes (en association avec l'œstrogène) et du rétrocontrôle négatif de la prolifération cellulaire (**L. Wang et Di 2014**). Dans un grand nombre de tumeurs mammaires TN (i) la présence de mutations homozygotes délétères, (ii) d'une hyper méthylation du promoteur du gène, ou encore (iii) d'anomalies dans la régulation épigénétique des gènes codant pour les protéines BRCA1 ou BRCA2, ont été retrouvés (**Telli et al. 2018**). Ces anomalies sont désignées par un phénotype appelé « **BRCAness*** » (**Dhillon et al. 2016**). Elles surviennent le plus souvent dans un contexte de cancer sporadique, mais elles peuvent également être retrouvées dans un contexte héréditaire. Par ailleurs,, plus de 85% des cancers du sein survenant dans un contexte de mutation héréditaire du gène BRCA1, présenteraient également les caractéristiques immunophénotypiques, morphologiques et moléculaires des tumeurs mammaires TN (**Rouleau et al. 2012**). En effet, le nombre de cancers du sein dont la survenue est favorisée

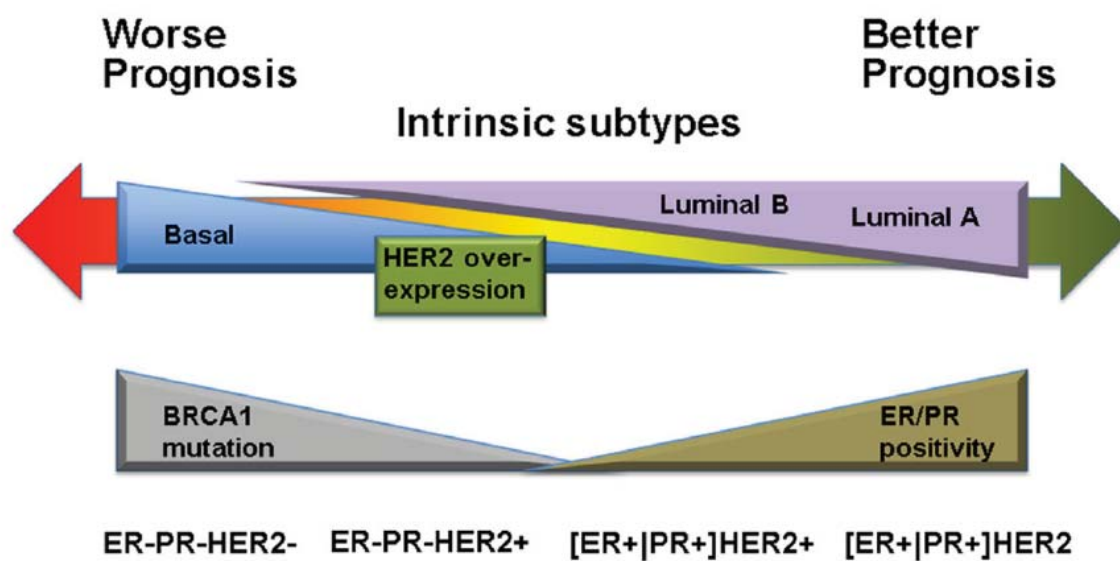


Figure 35 : Pronostique des sous-types intrinsèques de tumeurs mammaires.
Les tumeurs Triple-négatives sont de très mauvais pronostique (Dai, 2015)

par un contexte héréditaire est estimé entre 5 et 10%. Ils sont majoritairement associés à la présence de mutations germinales sur les gènes BRCA1 ou BRCA2 chez les patientes (**Dhillon et al. 2016**). Ces mutations germinales sont également fréquemment retrouvées chez les apparentés sains à l'état hétérozygote. Elles seraient synonymes d'une augmentation du risque de développements précoces de tumeurs mammaires TN (de 40 à 87%) ou de cancers de l'ovaire (de 45 à 60% pour BRCA1 et de 11 à 35% pour BRCA2). En effet, ce risque est augmenté par la plus forte probabilité de survenue d'un deuxième événement mutationnel sur ces deux gènes d'intérêt au sein des cellules pré-malignes mammaires ou ovariennes. Ainsi, la forte prévalence de ces mutations germinales spécifiques chez les porteuses de tumeurs mammaires TN ou de cancers ovariens a poussé les cliniciens à développer des stratégies de prises en charge spécifiques pour les apparentés sains. Des consultations d'Oncogénétique sont proposées depuis plusieurs années aux patientes et à leur apparentés, afin de déterminer le statut mutationnel de chacun des membres de la famille pour BRCA1 et BRCA2. Ces recherches, réalisées par séquençage, permettent de mettre en place des mesures prophylactiques pour les personnes « à risque » prenant la forme de suivis réguliers par radiographies ou échographies et des mastectomies et/ou ovariectomies préventives (**Gladieff et al. 2017; Kwiatkowski et al. 2016**). Par ailleurs, une étude récente a démontré que 4% des patientes atteintes de tumeurs TN présentaient également des mutations germinales sur d'autres gènes associés à la réparation de l'ADN (**Telli et al. 2018 ; Lord et Ashworth 2016; Ollier et al. 2015; Dhillon et al. 2016**). La recherche de mutations sur ces gènes permettrait d'étendre le panel des tumeurs *BRCAness* et donc d'élargir le screening effectué en Oncogénétique*. Ces stratégies permettraient d'améliorer la prévention et permettre le développement de nouvelles cibles thérapeutiques (**Rouleau et al. 2012**).

IV) Les traitements des cancers du sein Triple-Négatifs

Les tumeurs mammaires TN sont particulièrement agressives et généralement de mauvais pronostic en comparaison des autres sous-types de tumeurs mammaires (**Anders et al. 2016; Anders et Carey 2009; Bianchini et al. 2016**)(**Figure 35**). Quel que soit leur stade, elles sont représentées par une forte capacité proliférative. Cette caractéristique les

sensibilise dans un premier temps aux traitements néoadjuvants ou adjuvants classiques ainsi qu'à la radiothérapie.

En effet, la radiothérapie est utilisée dans le traitement des cancers mammaires de type TN, en curatif ou en palliatif. Par exemple, des séances de curage* après chirurgie mammaires sont réalisées, afin de limiter le risque de récurrence. Le bénéfice de ces séances a été démontré sur des patientes jeunes chinoises (<40 ans) atteintes de tumeurs mammaires TN localement avancées de stade 1 et 2. Une amélioration de la survie sans progression et de la survie globale était détectée chez ces patientes, en comparaison d'une mastectomie simple. Une autre étude effectuée chez les femmes atteintes de tumeurs TN métastasées de stade 3 et 4 a démontré une nette amélioration de la survie sans récurrence locorégionale et de la survie sans récurrence après radiothérapie post-mastectomie (**Vinh-Hung et Verschraegen 2004; Bhoo-Pathy et al. 2015**). Par ailleurs, les tumeurs portant des mutations sur **BRCA1** ou **BRCA2** seraient plus sensibles aux radiations ionisantes et aux régimes de chimiothérapies alkylantes. Ceci est dû à une forte instabilité génétique de réparation de l'ADN, dans ces tumeurs (**Bhoo-Pathy et al. 2015**). La radiothérapie est également fréquemment utilisée pour le traitement des métastases, notamment cérébrales, par des méthodes de radio chirurgie, par exemple (**Brown et al. 2017; El Shafie et al. 2018**).

Suite à ces traitements, une proportion non négligeable de patientes atteintes de tumeurs localisées ou localement avancées présentera des réponses pathologiques complètes. Quant au taux de survie, il serait comparable aux autres sous-types de tumeurs mammaires hormonodépendants, voire supérieurs (30 à 40%) (**Lehmann et al. 2016**). Cependant, pour l'autre moitié des patientes, un échec thérapeutique surviendra rapidement par le développement d'un foyer de cellules résistantes, entraînant à très court terme le développement de métastases (**Duma et al. 2018**). Pour ces patientes, le risque métastatique est douze fois plus élevé que pour celles atteintes d'autres sous-types du cancer du sein (**Lehmann et al. 2016**). Cet échec thérapeutique a par ailleurs été intitulé le « paradoxe triple-négatif » (**Carey et al. 2007; Anders et al. 2016; Haupt, Ro, et Schwartz 2010**). De plus, dans la mesure où les tumeurs TN n'expriment pas les récepteurs d'intérêt des thérapies antihormones (ER-, PR-, HER2 N), la stratégie thérapeutique pour ce type de cancer est très

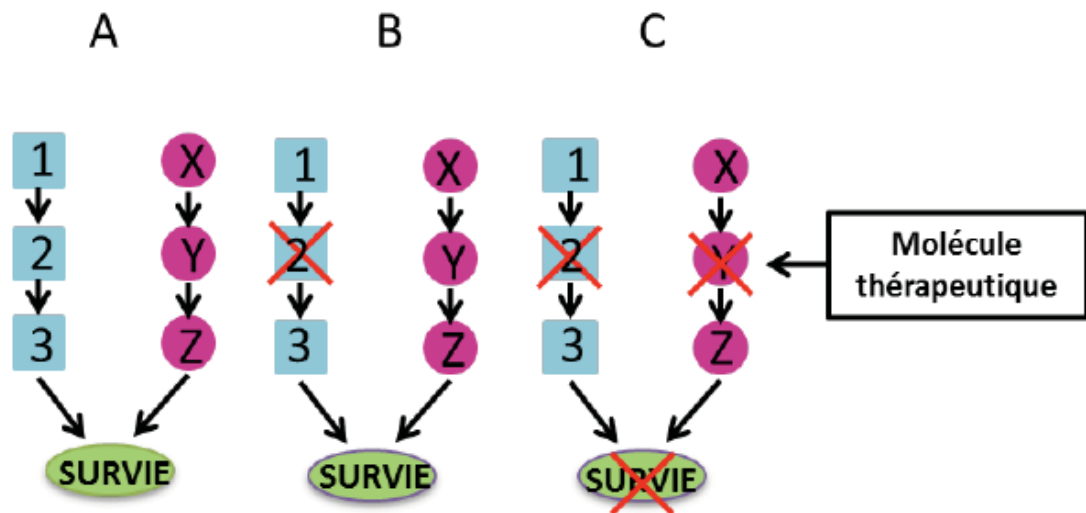


Figure 36 : Concept de létalité synthétique (d'après Fang B., 2014)

A : Un processus cellulaire vital est régulé par deux voies cellulaires différentes composées respectivement de 1, 2, 3 et X, Y, Z.

B : L'inactivation de l'une des deux voies n'est pas suffisante pour entraîner l'arrêt du processus, celui-ci est toujours régulé par la voie toujours active.

C : Même cas qu'en B, mais la deuxième voie est alors inhibée par une molécule thérapeutique. La fonction cellulaire n'est plus régulée et n'assure plus son rôle, entraînant la mort de la cellule.

complexe (**Ismail-Khan et Bui 2010**). De ce fait, de nombreux efforts sont actuellement menés afin de mettre en place de nouvelles stratégies de traitements de ces tumeurs agressives, notamment le développement de thérapies ciblées. Parmi ces derniers, les inhibiteurs des récepteurs de type EGFR (**El Guerrab et al. 2016; Anders et al. 2016; Nakai, Hung, et Yamaguchi 2016**), les inhibiteurs de tyrosine kinase (ITK) (**El Guerrab et al. 2016, 2017**), les immunomodulateurs (**Anders et al. 2016**) et les inhibiteurs de la Poly-ADP-Ribose-Polymérase (anti-PARPs) (**Xin Wang et al. 2018; O'Sullivan et al. 2014**), sont en cours d'études précliniques et cliniques.

C) Les inhibiteurs de Poly-ADP-Ribose-Polymérase

La classe des inhibiteurs de Poly-ADP-ribose Polymérase (ou protéines PARPs) utilise les déficiences dans les systèmes de réparation de l'ADN, propres à certains types tumoraux. Le principe de fonctionnement de cette nouvelle classe thérapeutique est basé sur le concept de létalité synthétique*.

I) Le concept de létalité synthétique

Les inhibiteurs de Poly-ADP-ribose polymérase ou anti-PARPs ont été les premiers agents de thérapie ciblée exploitant le concept de la létalité synthétique à être développés et approuvés (**Deeks 2015; Farmer et al. 2005**). Ce concept, développé depuis une vingtaine d'années, repose sur l'interaction de deux processus d'intérêt biologique majeur. La perturbation de l'un ou de l'autre événement ne sera pas létale prise indépendamment pour la cellule. Par contre, si cette perturbation touche les deux processus de manière simultanée, il pourrait engendrer la mort cellulaire programmée (**Figure 36**) (**Bryant et al. 2005; Bhattacharjee et Nandi 2017; O'Neil, Bailey, et Hieter 2017; Ohmoto et Yachida 2017; Farmer et al. 2005**). Dans ce contexte, le rôle des inhibiteurs de PARPs, consisterait en un blocage des voies de réparation de l'ADN *Base Excision Repair* (**BER**) et **HR**, conduisant ainsi la cellule tumorale vers l'apoptose.

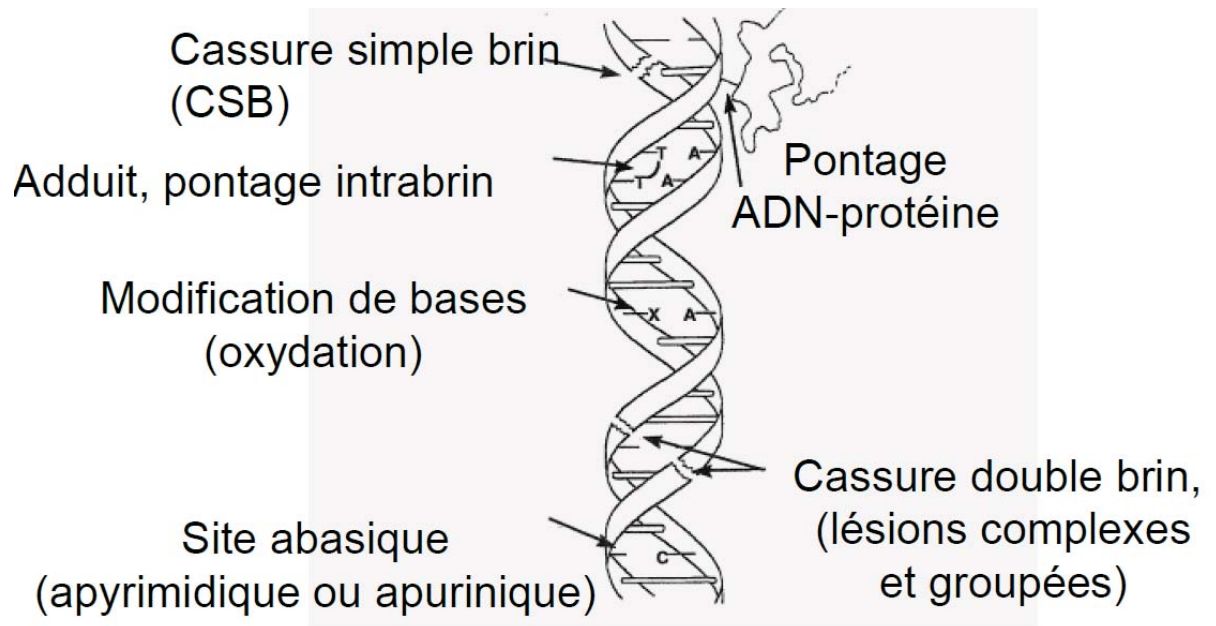


Figure 37 : Représentation des différentes lésions de l'ADN Simple et Double-brin

II) Les systèmes de réparation de l'ADN

Les systèmes de réparation de l'ADN jouent un rôle essentiel dans la survie cellulaire. Il en existe plusieurs, réparant ainsi différents types de lésions de l'ADN, plus ou moins fréquentes et critiques pour la cellule. Ces lésions sont générées suite à des agressions environnementales telles que l'exposition à des rayons ultraviolets, à des radiations ionisantes ou à des agents chimiques génotoxiques. Elles peuvent également être induites suite à des stress endogènes (O'Sullivan et al. 2014).

Les lésions générées peuvent affecter l'ADN simple-brin, engendrant différentes anomalies sous la forme de (i) **CSBs**, (ii) de mésappariements de bases, (iii) d'adduits et (iv) des pontages inter-brins (Rass et al. 2012). Ils peuvent également affecter l'ADN double-brin, sous la forme de **CDBs** (Figure 29).

(a) Réparation des cassures simple-brin (CSBs)

Les principaux systèmes de réparations des lésions de l'ADN simple-brin sont le système Mismatch Repair (**MMR**), le système Nucleotide Excision Repair (**NER**) et le système Base Excision Repair (**BER**).

❖ Le système de réparation par excision de base (*Base Excision Repair*, **BER**)

Le système **BER** répare les CSBs de l'ADN, les bases endommagées oxydées ou encore les sites abasiques* (Seeberg, Eide, et Bjørås 1995)(Figure 37). Les lésions sont détectées par la protéine PARP1 et excisées par des endonucléases. Par la suite, la protéine PARP1 permet la formation de chaînes de poly-ADP-riboses. Ces longues chaînes d'Adénosine Diphosphate Ribose permettent le recrutement et la fixation sur les sites lésés des protéines impliquées dans la réparation, selon deux systèmes : (i) le système de réparation par *patch* court (« *short-*

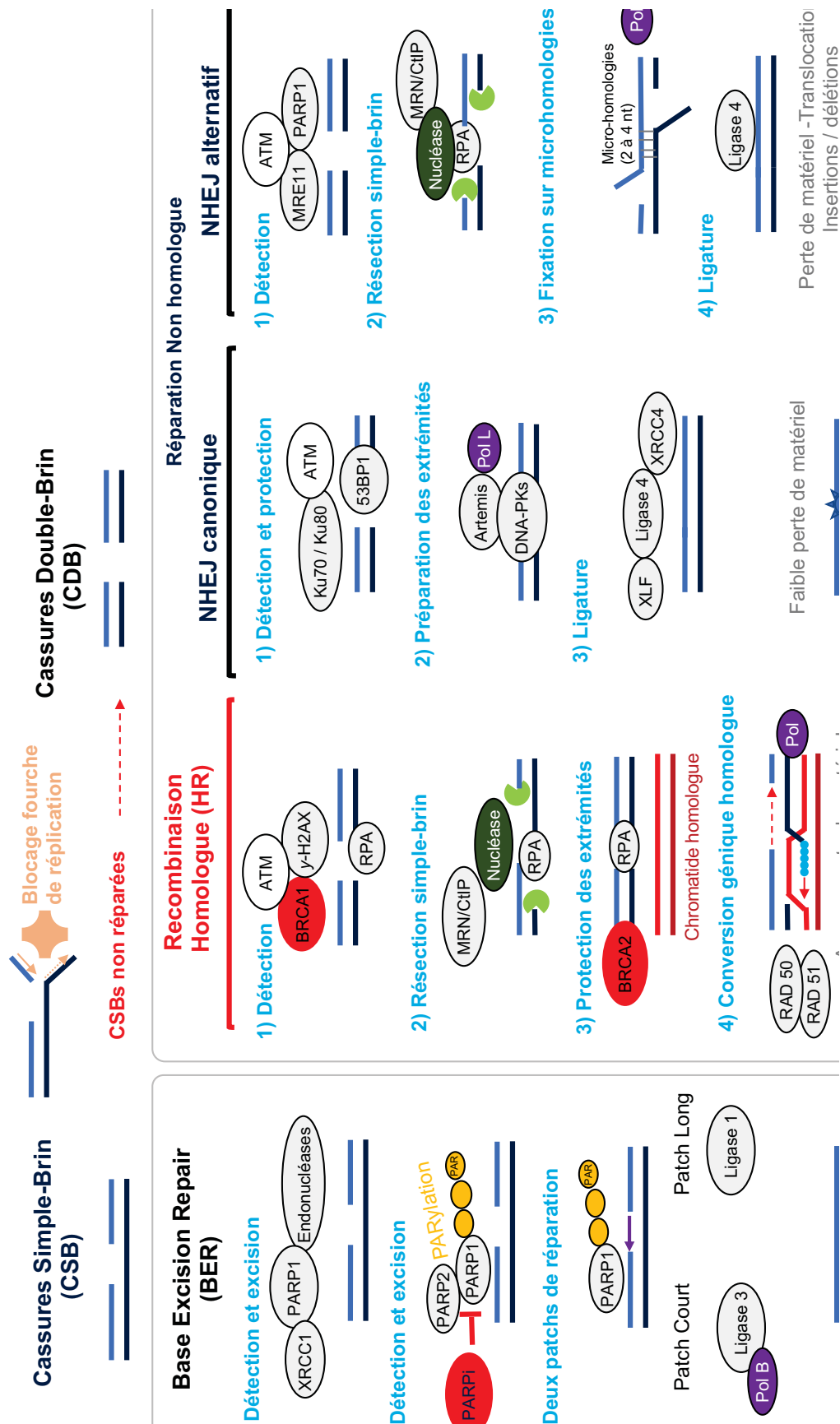


Figure 38 : Schéma récapitulatif des étapes et protéines impliquées dans les systèmes de réparation de l'ADN BER, HR et NHEJ (adapté de Rass 2012, Williams 2014, Mansour 2010 et Köttl 2014)

patch repair ») médié par XRCC1, la pol-béta et la ligase 3, et (ii) le système de *patch* long (« *long-patch repair* »), médié par la ligase 1 (**Figure 38**).

Le système **BER** est le système le plus sollicité dans la cellule pour la réparation des lésions simple-brin. Il est très fidèle et efficace. En effet, il répare plus de 20 000 CSBs par jour et par cellule, et permet de prévenir la survenue de mutations délétères ou la dégénérescence des CSBs en CDBs par explosion de la fourche de réplication (**Bryant et al. 2005**). Il a été démontré que la perte d'activité du système **BER** pour la réparation de mutations sur des oncogènes ou des gènes suppresseurs de tumeurs contribuait au développement tumoral (**Atamna, Cheung, et Ames 2000**).

(b) Réparation des cassures double-brin (CDBs)

Les CDBs de l'ADN, induites par des blocages de la fourche de réplication ou des dommages exogènes, sont quant à elles réparées par deux principaux systèmes : le système de réparation des CDBs par recombinaison homologue, et le système de recombinaison non homologue **NHEJ** (*Non Homologous End Joining*).

❖ **Le système de réparation des CDBs par recombinaison homologue (HR)**

Le système **HR** répare les CDBs en utilisant comme matrice de réplication le chromosome homologue à celui lésé. Un grand nombre de protéines sont impliquées dans ce mécanisme qui s'effectue en plusieurs étapes (**Rass 2012**). Tout d'abord, les CDBs sont détectées par les protéines BRCA1 et l'histone H2AX. La fixation de ces protéines déclenche le mécanisme de résection d'une large région d'ADN simple-brin (**Rass et al. 2012**). Cette résection est initiée par le complexe MRN/CtIP (constitué des protéines Mre11 et Rad50 entre autres), et finalisée par des nucléases. Afin d'éviter des translocations rendues possibles par la vulnérabilité du mono brin d'ADN, les extrémités saillantes 3' sont protégées par la protéine

RPA, qui sera déplacée par la protéine BRCA2 au moment de la réparation. Cette dernière recrute ensuite la protéine RAD51, qui va permettre l'invasion du mono brin d'ADN saillant sur le brin homologue et initier la conversion génique par l'ADN polymérase (**Figure 38**).

Le système **HR** est effectif seulement durant les phases S et G2 du cycle cellulaire, lorsque la chromatine homologue est accessible pour l'ADN polymérase (**Ohmoto et Yachida 2017; Bryant et al. 2005**). Ce mécanisme est très fiable. Il permet de limiter la perte de matériel génétique et ainsi maintenir l'intégrité génomique.

❖ Le système de réparation par recombinaison non homologue (NHEJ)

La réparation des CDBs par recombinaison non homologue se compose de deux mécanismes distincts : le **système NHEJ canonique (C-NHEJ)**, et le **système NHEJ alternatif (alt-NHEJ)**. Contrairement au système **HR**, ces deux systèmes ne requièrent pas de séquence homologue pour la réparation des CDBs. En effet, le système **C-NHEJ** répare les CDBs par suture directe des extrémités de chaque brin grâce à deux complexes protéiques principaux : les kinases ADN-dépendantes DNA-PKcs associées aux hétérodimères Ku70 et Ku80, et la ligase IV accompagnée de ces cofacteurs XRCC4 et XLF. La cassure double-brin est dans un premier temps détectée par les hétérodimères Ku70/80 qui forment un complexe avec les DNA-PKcs (**Williams et al. 2014**). Par ailleurs, la protéine 53BP1 se fixe aux extrémités des cassures afin de les protéger de la résection. Ensuite, lorsque la cassure double-brin présente des extrémités saillantes ou non compatibles, les nucléotides terminaux sont modifiés ou éliminés par des phosphokinasases ou des nucléases telles que PNK ou le complexe Artemis. Ensuite, les séquences manquantes sont reconstituées par l'ADN-polymérase lambda, et les deux brins sont finalement ligaturés grâce à XRCC4 couplée à la ligase IV et le cofacteur XLF (**Mansour, Rhein, et Dahm-Daphi 2010**). Ce système est moins fidèle que le système **HR**. En effet, de petites délétions ou insertions peuvent être générées à l'endroit de la cassure. Néanmoins, le génome contenant un grand nombre de séquences d'ADN non codantes, ces altérations microscopiques à l'échelle du génome ne font pas du système **C-NHEJ** un

phénomène très mutagène. Il permet en outre de maintenir l'intégrité chromosomique et donc préserver la stabilité génomique (**Figure 38**).

Par ailleurs, le deuxième système de réparation des cassures par ligature (système alternatif **alt-NHEJ**), récemment mis en évidence est beaucoup plus mutagène. En effet, dans ce système, les CDBs sont détectées grâce à la protéine PARP1. Puis, le complexe MRN/CtIP associé à des nucléases permet une résection large de l'ADN simple-brin. Les extrémités saillantes sont protégées par la protéine RPA. Ensuite, le système alternatif utilise comme séquence d'ancrage des micro homologies distantes de part et d'autre de la cassure double-brin (**Figure 38**). Ce mécanisme engendrerait de grandes délétions, et des translocations (**Rass et al. 2012; Kötter et al. 2014; Mansour, Rhein, et Dahm-Daphi 2010**). Le système **alt-NHEJ** est par ailleurs responsable de la commutation de classe des immunoglobulines et la recombinaison V(D)J, deux étapes permettant d'établir le répertoire immunitaire dans toute sa diversité (**Mansour, Rhein, et Dahm-Daphi 2010; Rass et al. 2012**).

Les deux systèmes de réparation **HR** et **NHEJ** agissent en parallèle pour la réparation de CDBs, et leur efficacité est primordiale pour le maintien de la survie cellulaire. En effet, les CDBs de l'ADN sont beaucoup moins fréquentes que les **CSBs** (40 CDBs vs 20 000 **CSBs** par jour et par cellule), mais sont extrêmement létales pour la cellule lorsqu'elles s'accumulent. En effet, après un certain nombre de cycles de réplication en présence de cassures non réparées, des mutations délétères ou des translocations peuvent survenir, augmentant ainsi l'instabilité génomique. Celle-ci peut atteindre des niveaux très élevés engendrant soit la promotion de la carcinogénèse (**Ohmoto et Yachida 2017**), soit la mort des cellules cancéreuses par apoptose (**J.-M. Lee, Ledermann, et Kohn 2014; Bhattacharjee et Nandi 2017**). Il s'agit donc d'un équilibre instable permanent entre stabilité et variabilité du génome, pouvant être utilisé au profit de la médecine, comme outils thérapeutique.

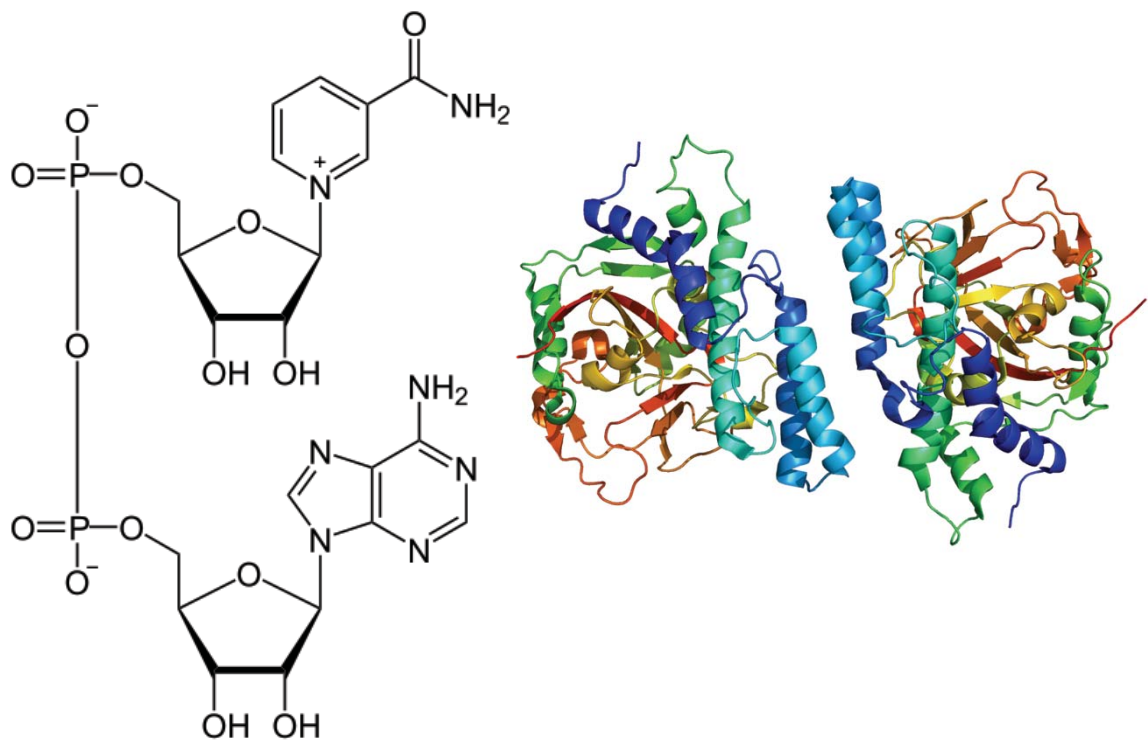


Figure 39 : Structure de la protéine Poly-ADP-ribose polymérase 1 (vue plane et en 3D)

III) Les protéines PARPs

Les poly-ADP-ribose polymérases, découvertes en 1963, sont une famille de 17 protéines nucléaires présentant une activité enzymatique de catalyse de l'ADP-ribosylation dépendante du NAD⁺ (O'Sullivan et al. 2014)(Figure 39). Elles sont impliquées dans un grand nombre de processus biologiques tels que la différenciation cellulaire, l'inflammation, la mitose, le métabolisme cellulaire et la réparation de l'ADN. La protéine PARP1, majoritaire, est impliquée dans le mécanisme de réparation des CSBs **BER**. En effet, elle se fixe sur les CSBs grâce à son extrémité N-terminale en doigt de zinc et active la polymérisation des chaînes d'ADP-riboses par un phénomène de PARylation. Cette chaîne de poly-ADP-riboses permet le recrutement des protéines effectrices de la réparation des CSBs que sont XRCC1 (*X-ray Repair Cross-Complementing protein 1*), l'ADN polymérase bêta, l'ADN ligase 1 ou 3, qui permettent la synthèse et la ligature du brin lésé. La protéine PARP2, représentant 5 à 10% de l'activité totale des protéines PARPs, est également impliquée dans la réparation des cassures simple brin, en étroite collaboration avec la PARP1 (Ohmoto et Yachida 2017). Par ailleurs, la protéine PARP1 est également impliquée dans les processus de réparation des CDBs **HR** et **NHEJ canonique et alternatif**. En effet, elle a un rôle activateur du système **HR** grâce à un contrôle positif sur la kinase ATM. Elle exerce également un rôle inhibiteur sur le système **NHEJ canonique**, par son effet répresseur sur les kinases ADN-dépendantes impliquées dans ce processus (O'Sullivan et al. 2014). De cette manière, la PARP1 est impliquée dans le fonctionnement de ces deux systèmes de réparation des CDBs (Mansour, Rhein, et Dahm-Daphi 2010). Par ailleurs, la protéine PARP1 permet de réduire le nombre de CDBs produites dans la cellule. En effet, elle détecte les fourches de réplication « bloquées » et elle recrute la protéine MRE11 pour induire une réparation par **HR** (O'Sullivan et al. 2014). Ainsi, la protéine PARP1 est essentielle au bon déroulement de la détection et de la réparation d'un grand nombre de cassures.

Par ailleurs, dans un grand nombre de tumeurs, et notamment les tumeurs mammaires TN, l'activité et/ou l'expression de PARP1 semble bien élevée, induisant ainsi des anomalies dans leurs systèmes de réparation de l'ADN, et plus précisément, le système de réparation

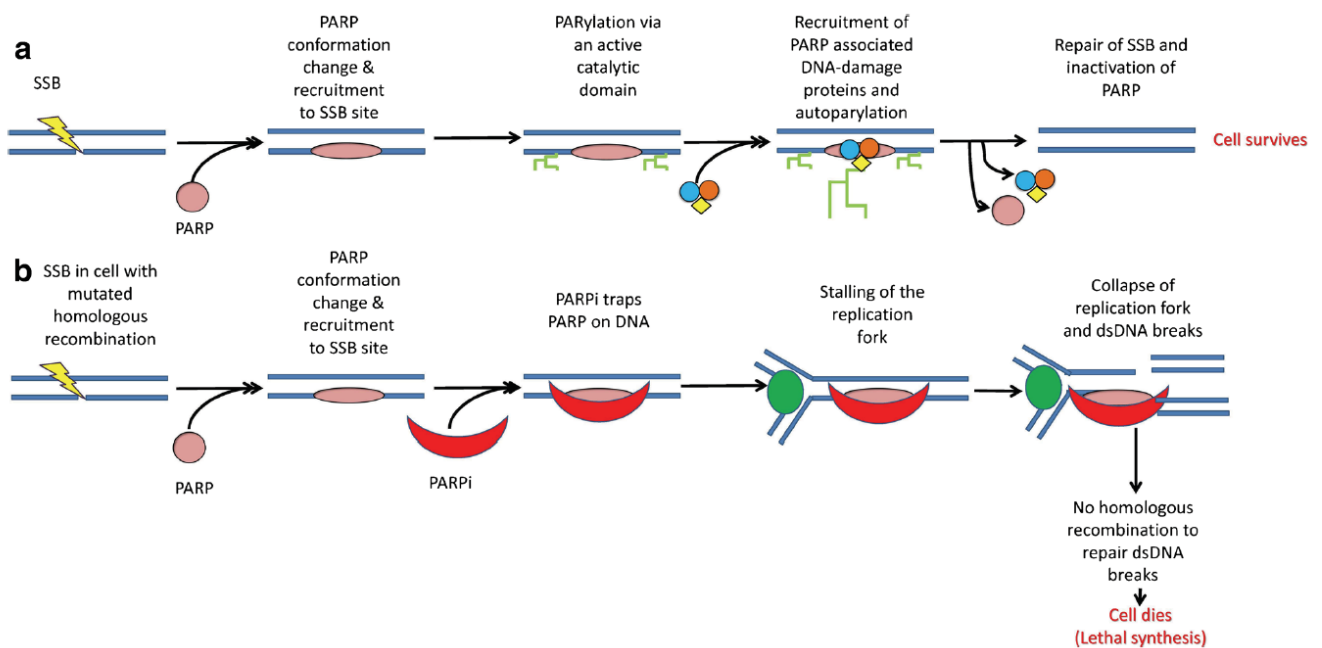


Figure 40 : Mécanisme d'action des inhibiteurs de PARP (PARPi). Les PARPi séquestrent les protéines PARP alors qu'elles sont fixées sur le site de cassure simple brin de l'ADN. Cette fixation empêche le phénomène de PARylation permettant le recrutement des protéines en aval de la cascade de réparation. Cet encombrement sur le site de la cassure va provoquer, à terme, l'explosion de la fourche de réplication et la génération d'une cassure double-brin (Duma, 2018).

HR. Ainsi, dans ces cellules tumorales, ce sont uniquement les systèmes de réparation **NHEJ** qui prennent le relai pour la réparation des CDBs.

Dans ce contexte, la déficience du système **HR** dans ces tumeurs peut être utilisée comme « un processus » afin de développer une approche de létalité synthétique. L'autre processus, serait l'inhibition des protéines PARP à l'aide des thérapeutiques ciblées appelées inhibiteurs de PARP1, induisant l'inhibition de la voie **BER** et par conséquent, une accumulation létale des CDBs dans le génome de ces tumeurs. C'est le principe même de la toxicité des **inhibiteurs de PARP** et la raison du développement des inhibiteurs de PARP1 et PARP2 et leur application en tant que thérapie ciblée.

IV) Mécanisme d'action des inhibiteurs de PARPs

Les inhibiteurs de PARPs ont la capacité de se fixer sur le site actif de la protéine PARP1, quand celle-ci est liée à l'ADN suite à la détection d'une lésion. La protéine PARP étant piégée, ne pourra pas accéder au site de la cassure de la chaîne de réparation, en aval. Cette action bloque alors le système de réparation des CSBs **BER** engagé, et engendrant ainsi l'explosion de la fourche de réplication et la formation d'un **CDB** (**Figure 40**).

V) Les inhibiteurs de PARP en développement

Les premiers travaux reportant l'action des inhibiteurs de PARP sur des cellules mutées pour les gènes BRCA1 ou BRCA2 datent de 2005 (**Farmer et al. 2005; Bryant et al. 2005**). Sur la base de ces observations, la preuve de concept de l'inhibition de PARP sur des tumeurs avancées ovariennes et mammaires déficientes pour BRCA1 ou 2 a été démontrée dans des études cliniques (**Telli et al. 2018**). De par leur intérêt dans l'induction de la létalité synthétique chez certaines tumeurs, de nombreux inhibiteurs de PARP ont été développés par les firmes pharmaceutiques. L'Olaparib (AstraZeneca/KuDOS), le Rucaparib (Pfizer/Clovis), le Vélaparib (Abbvie), le Niraparib (**Moore, Mirza, et Matulonis 2018**) (Merck/Tesaro) et le

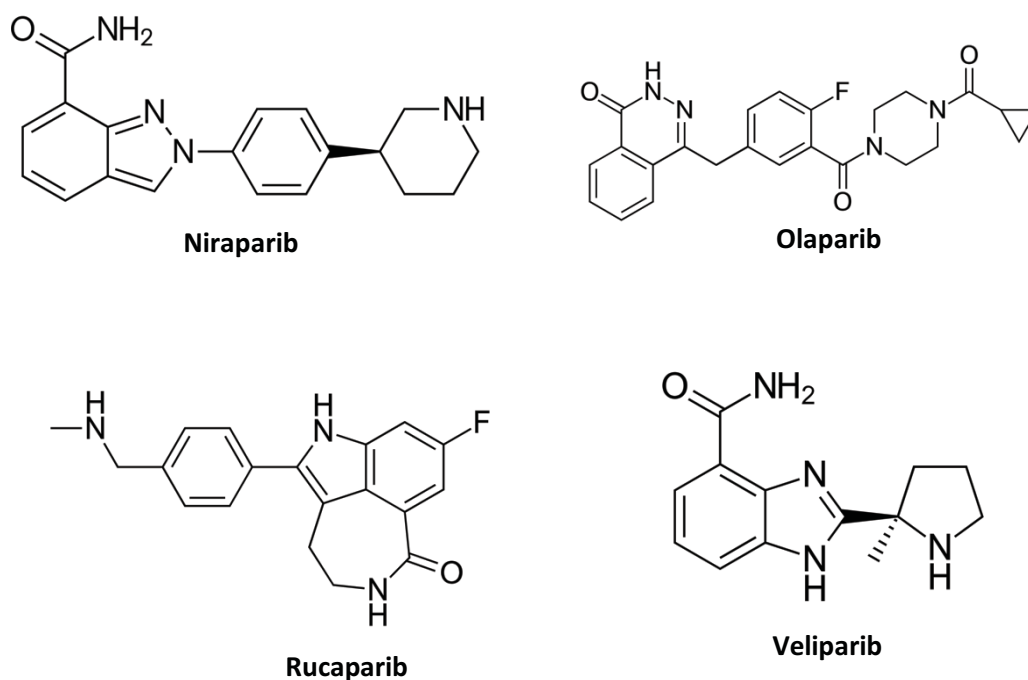


Figure 41 : Structure des différents anti-PARPs développés

PARP inhibitor	Combination agents	Phase	Types of malignancies	NCT identifier
Olaparib	AZD2014 (mTORC 1/2 inhibitor) or AZD5363 (AKT inhibitor)	I	Breast cancer, malignant female reproductive system tumors	NCT02208375
Olaparib	Prexasertib	I	Solid tumors	NCT03057145
Olaparib	AZD2281, AZD5363, AZD1775 or AZD2014	II	Tumors	NCT02576444
Olaparib	AT13387 (heat shock protein 90 inhibitor)	I	Ovarian cancer, fallopian tube cancer, peritoneal cancer and TNBC	NCT02898207
Olaparib	Cediranib, MEDI4736 (anti-PD-L1 antibody)	I/II	Lung cancer, breast cancer, ovarian cancer, colorectal cancer, prostate cancer and TNBC	NCT02484404
Olaparib	Temozolomide	I	Ewing sarcoma	NCT01858168
Olaparib	AZD1775 (tyrosine kinase WEE1 inhibitor)	I	Ovarian cancer, breast cancer and SCLC	NCT02511795
Olaparib	Temozolomide	I	Brain and central nervous system tumors	NCT01390571
Olaparib	CRLX101 (nanoparticle camptothecin)	I/II	SCLC	NCT02769962
Olaparib	Neoadjuvant carboplatin and paclitaxel	II/III	TNBC and germline BRCA-positive breast cancer	NCT03150576
Talazoparib	Decitabine	I/II	Acute myeloid leukemia	NCT02878785
Talazoparib	Temozolomide	I/II	Solid tumors	NCT02116777
Veliparib	VX-970 (ATR) and cisplatin	I	Tumors	NCT02723864
Veliparib	Intraperitoneal floxuridine	I	Fallopian tube cancer, ovarian cancer and primary peritoneal cancer	NCT01749397
Veliparib	Carboplatin and paclitaxel	III	BRCA-mutant breast cancer	NCT02163694
Veliparib	FOLFIRI or modified FOLFIRI	II	Pancreatic cancer	NCT02890355
Veliparib	Carboplatin and paclitaxel	III	Ovarian cancer, fallopian tube cancer and peritoneal cancer	NCT02470585
Veliparib	Topotecan hydrochloride	I/II	Solid tumors	NCT01012817
Niraparib	Temozolomide or irinotecan	I	Ewing sarcoma	NCT02044120
Niraparib	Pembrolizumab	I/II	Ovarian cancer, fallopian tube cancer, peritoneal cancer and TNBC	NCT02657889
Fluzoparib	Apatinib and paclitaxel	I	Gastric cancer	NCT03026881
Fluzoparib	Apatinib	I	Ovarian cancer and TNBC	NCT03075462
BGB-290	BGB-A317 (anti-PD-1 monoclonal antibody)	I	Solid tumors	NCT02660034

Abbreviations: FOLFIRI, folinic acid/fluorouracil/ irinotecan; NCT, National Clinical Trial; PD-1, programmed death-1; PD-L1, programmed death-ligand1; TNBC, triple-negative breast cancer; SCLC, small cell lung cancer.

Figure 42 : Différents anti-PARPs en cours de développement et d'essai clinique (Ohmoto, 2017)

Talazoparib (Lead/Biomarin/Medivation/Pfizer) (**Duma et al. 2018**) sont les molécules plus représentatives et qui pour la plupart, sont en cours de recherche clinique (**Figure 41 et 42**). L'Olaparib, le Rucaparib et le Niraparib ont été approuvés en 2014 aux USA et en Europe pour le traitement des cancers ovariens avancés porteurs de mutations germinales sur les gènes BRCA1/2 (**Ohmoto et Yachida 2017**). Par la suite en 2018, l'Olaparib a obtenu une autorisation de la FDA pour le traitement des cancers du sein métastatiques (« Approved Drugs - FDA Approves Olaparib for Germline BRCA-Mutated Metastatic Breast Cancer » s. d.). L'Olaparib, le Véliparib et le Talazoparib sont également à l'essai en monothérapie* ou en combinaison avec des thérapies cytotoxiques, des thérapies ciblées ou de l'immunothérapie. Ces combinaisons sont en cours d'étude pour le traitement d'un certain nombre de tumeurs solides présentant également des déficiences dans les systèmes **HR**, telles que du pancréas, du poumon (non à petites cellules), des métastases cérébrales, des cancers tête et cou et des mélanomes, (**Xin Wang et al. 2018; Duma et al. 2018; O'Sullivan et al. 2014**). En effet, environ 5% des mélanomes cutanés sont porteurs de mutations germinales de BRCA1/2 et 5 à 19% des cancers pancréatiques familiaux sont attribués à ces mêmes mutations. De mêmes, des mutations germinales sur BRCA1 sont retrouvées dans environ 1% des cas de cancer de la prostate (**Ohmoto et Yachida 2017**). En outre, 31 essais utilisant des anti-PARPs ont été répertoriés en 2018 sur le site clinicaltrials.gov.

L'Olaparib® est un agent thérapeutique développé par la firme AstraZeneca sous le nom commercial de Lynparza®. Cette molécule est un inhibiteur des protéines PARP-1, PARP-2 et PARP-3 (**Deeks 2015; Marchetti et al. 2012; « Approbation des comprimés Lynparza® (olaparib) pour le traitement d'entretien du cancer de l'ovaire, Indépendamment du statut mutationnel de BRCA » s. d.**). Des études précliniques ont démontré la capacité d'inhibition de croissance de l'Olaparib sur un panel de lignées cellulaires tumorales *in vitro* et également sur des modèles *in vivo*, que ce soit en monothérapie ou en combinaison avec des chimiothérapies conventionnelles. De plus, des modèles murins déficients pour BRCA1 et 2 ont présenté une croissance tumorale retardée, lorsqu'ils étaient traités avec de l'Olaparib, en plus des sels de platine, et en comparaison du bras sels de platine seuls (**J.-M. Lee, Ledermann, et Kohn 2014**). Ces études précliniques suivies d'études cliniques multiples ont permis l'obtention d'une autorisation de mise sur le marché du Lynparza en Décembre 2014,

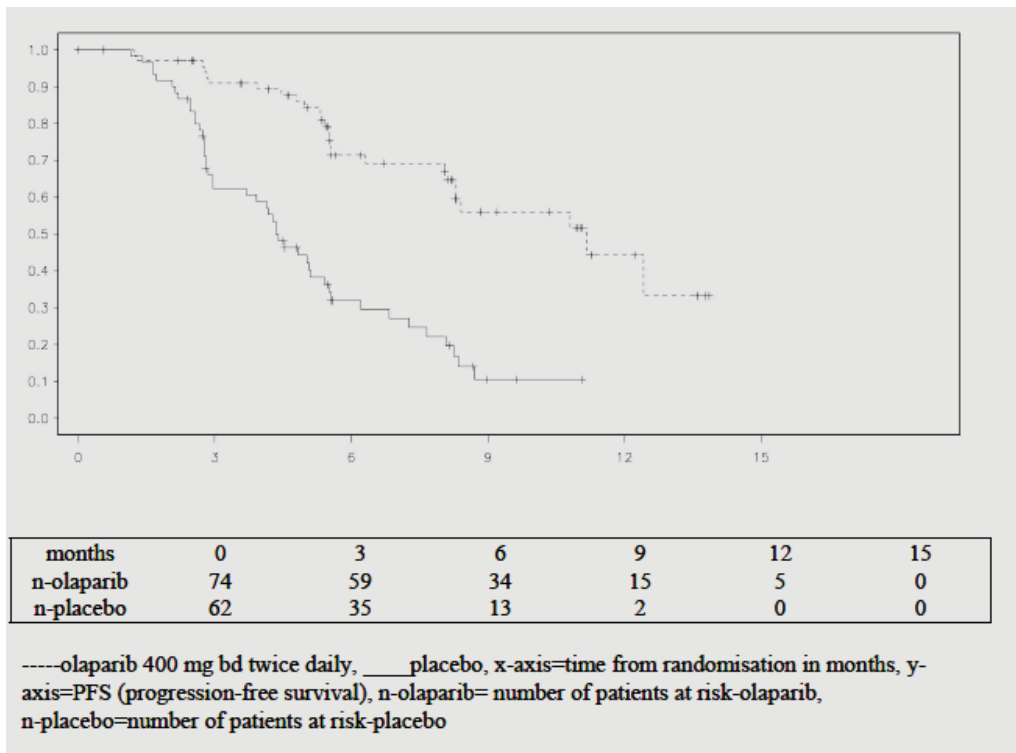


Figure 43 : Figure de Kaplan Meier démontrant l'efficacité de l'Olaparib sur des tumeurs ovariennes mutées BRCA1/BRCA2 en termes de survie sans progression (source= indication de l'Olaparib/Lynparza)

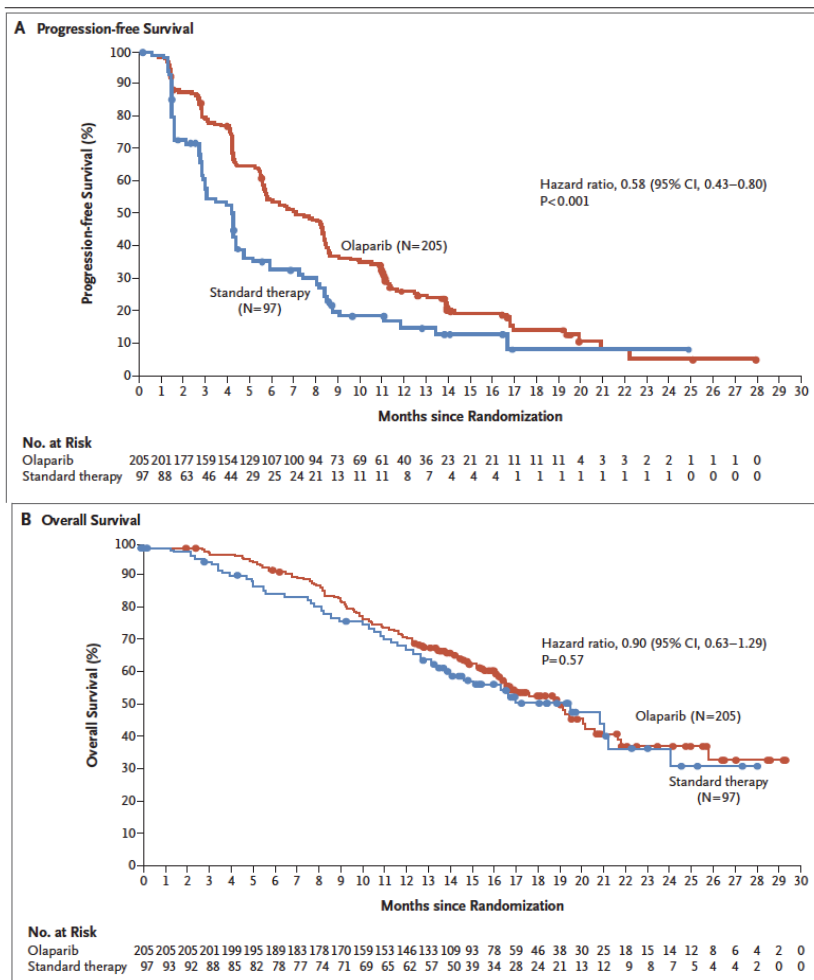


Figure 44 : Essai clinique de phase III OlympiAD (Astra Zeneca) pour l'évaluation de l'efficacité de l'Olaparib sur des tumeurs mammaires métastatiques porteuses de mutations germinales sur BRCA1/2 – Figures de Kaplan-Meier pour la survie sans progression (A) et la survie globale (B) (Robson, 2017)

pour la prise en charge thérapeutique des cancers de l’ovaire, des trompes de Fallope, et des cancers péritonéaux primitifs présentant des mutations germinales et/ou somatiques sur les gènes BRCA1 et/ou 2. L’Olaparib est en l’occurrence indiqué pour le traitement de maintenance des cancers ovariens en réponse complète ou partielle à la chimiothérapie à base de sels de platine en monothérapie. La prise d’Olaparib se fait par voie orale, et la posologie recommandée est de 8 comprimés de 50 mg (soit 400 mg par prise) deux fois par jour (soit 800 mg/jour). Par ailleurs, une posologie de seulement 400 mg/jour a permis d’obtenir, sur 136 patientes BRCA-mutées, une augmentation de la survie sans progression de 6,9 mois en comparaison du *placebo* (**Figure 43**) (Deeks 2015; Marchetti et al. 2012; « **Approbation des comprimés Lynparza® (olaparib) pour le traitement d’entretien du cancer de l’ovaire, Indépendamment du statut mutationnel de BRCA** » s. d.).

Pour le traitement des cancers du sein, un très grand nombre d’essais cliniques sont réalisés avec l’Olaparib. En particulier, l’étude de **phase III OlympiAD** (Astra Zeneca NCT02000622) a étudié l’efficacité de l’Olaparib en monothérapie pour le traitement des cancers du sein métastatiques HER2 – porteurs de mutations germinales de BRCA1 ou BRCA2 et ayant reçu pas plus de deux protocoles de chimiothérapie pour traiter la maladie métastatique (Le et Gelmon 2018; Gyawali 2017; Robson et al. 2017). Les patientes étaient traitées soit avec l’Olaparib (2 fois 300 mg par jour pour 205 patientes) soit avec un autre agent de chimiothérapie conventionnelle pour le bras contrôle (capécitabine, éribuline ou vinorelbine, pendant des cycles de 21 jours pour 97 patientes). La médiane de survie sans progression a été significativement allongée dans le groupe Olaparib (7,0 mois) en comparaison de la thérapie conventionnelle (4,2 mois) (HR 0,58, intervalle de confiance 95%, 0,43 – 0,80, $p < 0,001$) (**Figure 44**). De plus, le risque de progression de la maladie ou de mort était 42% plus faible avec l’Olaparib administré en monothérapie qu’avec la thérapie standard. Par ailleurs, les effets secondaires de haut grade (grade 3) étaient moins fréquents dans le groupe Olaparib (37%) que dans le groupe standard (51%). En revanche, aucun bénéfice sur la survie globale n’a été démontré avec l’Olaparib en comparaison du traitement standard.

Néanmoins, suite à ces résultats encourageants, la FDA a étendu en Janvier 2018 l’indication de l’Olaparib au traitement des cancers du sein métastatiques HER2- et ER+/-

Grade 3–4 adverse events	Olaparib (n=298)⁴²	Talazoparib (n=71)⁴⁷	Rucaparib (n=204)⁵⁰	Veliparib (n=50)⁵²	Niraparib (n=367)⁵⁴
Nausea	0%	0%	4%	4%	3%
Vomiting	2%	Not reported	2%	0%	2%
Fatigue	6%	Not reported	9%	Not reported	8%
Abdominal pain	6%	Not reported	2%	Not reported	1%
Thrombocytopenia	Not reported	18%	2%	2%	34%
Anemia	17%	23%	22%	0%	25%
Neutropenia	Not reported	10%	7%	2%	20%
Hypertension	Not reported	Not reported	Not reported	Not reported	8%
Increased AST/ALT	Not reported	Not reported	12%	Not reported	Not reported

Abbreviations: AST, aspartate transaminase; ALT, alanine aminotransférase; PARP, poly(ADP-ribose) polymerase.

Figure 45 : Effets secondaires des différents anti-PARPs lors des essais cliniques (Ohmoto, 2017)

porteurs de mutations germinales sur les gènes BRCA1 et BRCA2, ayant déclaré une hormonorésistance et précédemment traités par chimiothérapie au stade néoadjuvant, adjuvant, ou métastatique (**Oncology News Burst s. d.; Robson et al. 2017**). Selon cette indication, la dose journalière d'Olaparib recommandée est de 600 mg (deux cachets journaliers de 300 mg).

VI) Effets secondaires des traitements anti-PARPs

Les anti-PARPs présentent un certain nombre d'effets secondaires, principalement de bas grade (1 et 2) tels que de la fatigue et des nausées. Certains patients ont développé des effets secondaires de haut grade tels qu'une hépatotoxicité ou des thrombocytopénies dans moins de 10 % des cas, notamment avec le Rucaparib et le Niraparib. Par ailleurs, la toxicité hématologique dépend du type d'anti-PARP administré. En effet, le Niraparib est l'agent le moins hématotoxique, avec moins de 2% d'effets secondaires répertoriés. Les effets secondaires les plus graves ont été des syndromes myélodysplasiques et des leucémies myéloïdes aigues (**Xin Wang et al. 2018**)(**Figure 45**).

L'Olaparib présente principalement une toxicité gastro-intestinale de bas grade (1 à 2), avec de fréquents cas de nausées, de vomissements, d'anorexie et de diarrhées. Par ailleurs, des toxicités hématologiques ont été répertoriées telles qu'une anémie, une thrombocytopénie ou des neutropénies, associées à de la fatigue (**Deeks 2015; Xin Wang et al. 2018; « Approbation des comprimés Lynparza® (olaparib) pour le traitement d'entretien du cancer de l'ovaire, Indépendamment du statut mutationnel de BRCA » s. d.**) (**Figure 45**).

VII) Biomarqueurs de l'efficacité des anti-PARPs : le phénotype « BRCAness »

Le principal biomarqueur de l'efficacité des anti-PARPs est la présence de mutations germinales et/ou somatiques sur les gènes BRCA1 et BRCA2. Également, (i) la perte

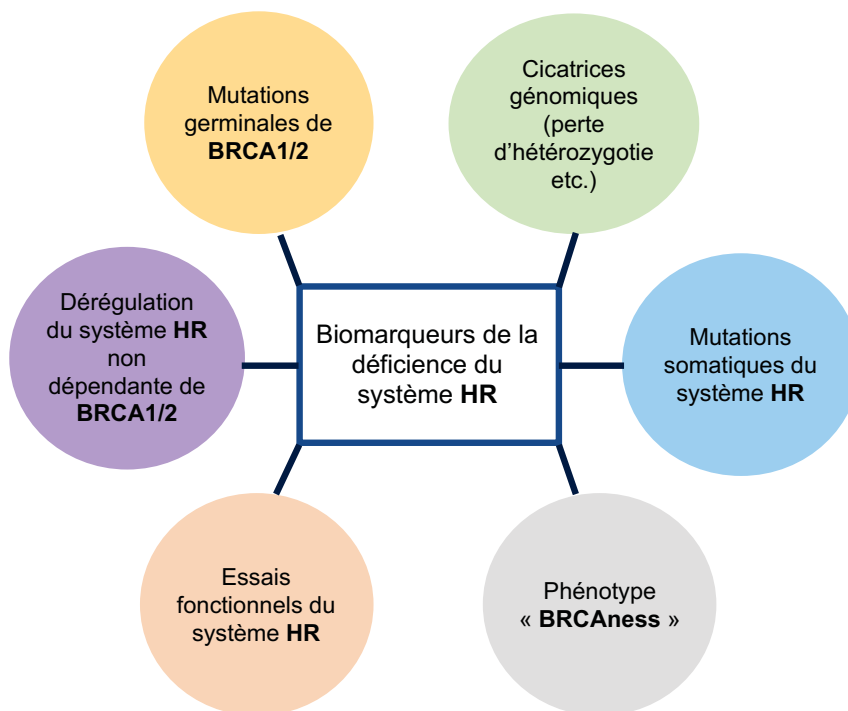


Figure 46 : Biomarqueurs de la déficience du système de réparation de l'ADN par Recombinaison Homologue (Homologous Recombination, HR) (Telli, 2018)

épigénétique* de la fonction de BRCA1 par la méthylation du promoteur, (ii) la surexpression du répresseur transcriptionnel interagissant avec BRCA2 (EMSY), (iii) la surexpression de la protéine AURKA impliquée dans la régulation du cycle cellulaire et (iv) un déséquilibre télomérique allélique peuvent être à l'origine de la sensibilité aux anti-PARPs (**Ohmoto et Yachida 2017; Ihnen et al. 2013**). Par ailleurs, il a été démontré que des tumeurs non mutées pour BRCA1/2 pouvaient tout de même présenter une sensibilité aux traitements par des anti-PARPs (**Telli et al. 2018**). Ainsi, d'autres analyses ont mis en évidence de nouveaux biomarqueurs de l'efficacité des anti-PARPs, tels que la présence de mutations sur RAD51, ATM, ATR, CHEK1, CHEK2, FANCD2, et FANCA (**Telli et al. 2018; Xin Wang et al. 2018**). Par ailleurs, l'amplification de MYC ou des mutations de P53 seraient également des marqueurs de déficience du système HR, tout comme une perte de PTEN. L'ensemble de ces paramètres définissent un périmètre de sensibilité aux anti-PARPs plus large que les simples mutations sur les gènes BRCA1/2, que l'on appelle le phénotype « **BRCAness** ». Ce phénotype est évaluable par différents scores, et notamment le HRD (« HR déficient »), qui permet de classer les tumeurs selon un grade de déficience des cellules tumorales pour le système HR (**Figure 46**). Ainsi, selon ce score, plus de 50% des tumeurs mammaires TN sont classées comme étant « **BRCAness** » et sont donc susceptibles de répondre aux thérapies anti-PARPs (Telli et al. 2018).

Malgré les outils de *scoring* et les avancées dans les classifications permettant de définir la sensibilité potentielle des tumeurs aux anti-PARPs, de nombreux obstacles émergent lors des essais précliniques et cliniques. Notamment, des problèmes de sélection, de planification et de dosage ainsi que des phénomènes de chimiorésistance limitant ainsi l'efficacité de ces traitements. (**Duma et al. 2018**).

VIII) La résistance aux anti-PARPs

(a) Résistance moléculaire

Certaines tumeurs mutées BRCA1/2 ne répondent pas au traitement par anti-PARPs. Une des premières explications de cet échec serait le rétablissement du système de

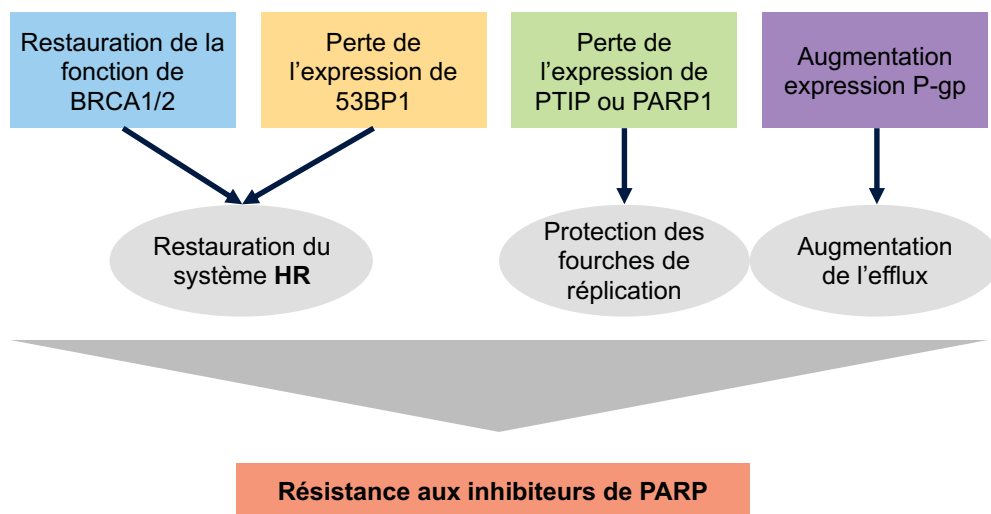


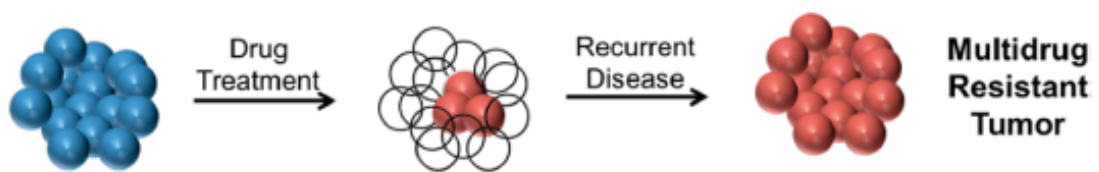
Figure 47 : Principaux mécanismes moléculaires ou cellulaires connus de la résistance aux anti-PARPs (Dhillon, 2016)

recombinaison homologue au sein de ces tumeurs, par la survenue de mutations secondaires restaurant partiellement l'activité des gènes BRCA1 ou BRCA2 (O'Neil, Bailey, et Hieter 2017; Dhillon et al. 2016). Par ailleurs, la perte d'activité de la protéine 53BP1, impliquée dans le système NHEJ a été identifiée comme facteur de résistance aux anti-PARPs. Le mécanisme de cette résistance n'est toutefois pas encore totalement identifié (Dhillon et al. 2016; Rottenberg et al. 2008). Un autre mécanisme serait la perte d'expression des protéines dégradant les fourches de réplication, telles que PTIP et MRE11. L'absence de dégradation des fourches, et donc leur persistance, serait un facteur de réduction de l'instabilité génomique, et donc de résistance aux anti-PARPs (Dhillon et al. 2016)(Figure 47).

(b)Résistance cellulaire

Cette absence de réponse des tumeurs TN pourrait également être expliquée par l'augmentation de l'efflux cytoplasmique des anti-PARPs à l'échelle cellulaire, notamment par une surexpression de certaines protéines de *Multidrug Resistance* (MDR) (Rottenberg et al. 2008; Dhillon et al. 2016; Lord et Ashworth 2013). En effet, plusieurs études *in vitro* et *in vivo* ont déjà mis en évidence (1) une augmentation de l'expression de la P-gp et de la BCRP suite au traitement par Olaparib (Dufour et al. 2015) et (2) une forte résistance aux anti-PARPs chez des souris porteuses de tumeurs BRCA1 et 2 déficientes pour lesquelles le niveau d'expression de P-gp était élevé (Rottenberg et al. 2008). En outre, l'Olaparib a été identifié comme substrat de la P-gp, et serait également un inhibiteur compétitif potentiel pour la pompe BCRP (O'Neil, Bailey, et Hieter 2017; Deeks 2015; « **Approbation des comprimés Lynparza® (olaparib) pour le traitement d'entretien du cancer de l'ovaire, Indépendamment du statut mutationnel de BRCA** » s. d.). La prise d'Olaparib pourrait alors induire une augmentation de l'exposition des autres substrats de ces pompes, et notamment des statines, de la digoxine et du méthotrexate. Le risque de toxicité de ces molécules serait donc accru suite à la prise de l'Olaparib (Deeks 2015; « **Approbation des comprimés Lynparza® (olaparib) pour le traitement d'entretien du cancer de l'ovaire, Indépendamment du statut mutationnel de BRCA** » s. d.). Par ailleurs aucune donnée ne décrit à ce jour l'implication de la protéine MRP7 dans la résistance aux anti-PARPs.

A Drug-induced selection (acquired resistance):



B Pre-existing (chemo-insensitive stem-like cancer cells):

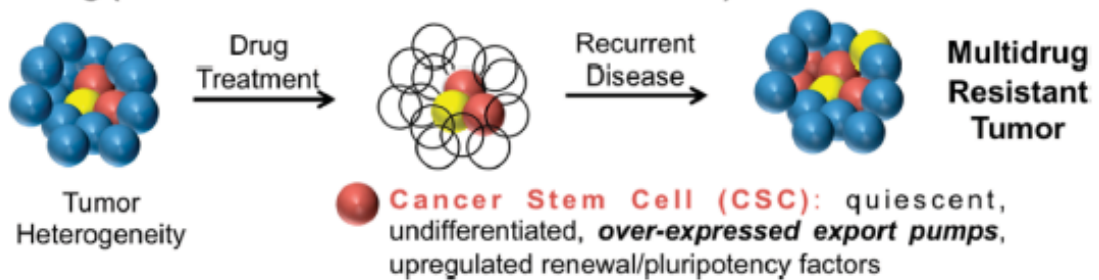


Figure 48 : Différents mécanismes de la chimiorésistance, pouvant être acquise (A) suite aux traitements ou innée (B) par la présence de cellules souches cancéreuses intrinsèquement résistantes (Vargas, 2014).

Ce dernier mécanisme de résistance *via* les protéines **MDR** fait donc l'objet d'un intérêt tout particulier dans les études précliniques, de par son fort risque de résistance croisée avec d'autres molécules antinéoplasiques.

D) La chimiorésistance

La chimiothérapie est l'une des voies principales du traitement du cancer qui a permis de faire reculer le taux de mortalité par cancer. Néanmoins, celui-ci reste encore très élevé, à cause des échecs thérapeutiques et notamment la chimiorésistance. Cette dernière se définit par la présence de mécanismes de résistance retrouvés chez les cellules cancéreuses, contre un très large panel de molécules chimiothérapeutiques (**Vargas et al. 2014**). Ce phénomène de chimiorésistance, pouvant être inné ou acquis suite aux traitements (**Figure 48**), est engendré par un panel de perturbations biochimiques et cellulaires multiples. La combinaison de ces anomalies à différents niveaux de la machinerie cellulaire génère une résistance à un panel varié de molécules thérapeutiques. En effet, ces phénomènes peuvent mettre en jeu :

1. Des perturbations de l'expression des protéines ou enzymes cibles par des dérégulations post-transcriptionnelles ou post-translationnelles, ou par des mutations délétères (**Bugde et al. 2017**).
2. Des perturbations de l'activation ou de la dégradation du médicament (**Bugde et al. 2017**).
3. Une inactivation constitutive de l'apoptose (mutations de P53) (**Bugde et al. 2017**)
4. Des anomalies du transport des molécules thérapeutiques par l'augmentation de leur efflux ou la diminution de leur pénétration (**Bugde et al. 2017; Vargas et al. 2014**).

Ce dernier processus de résistance intervient à l'échelle de la membrane cellulaire. Il implique principalement des pompes d'efflux transmembranaire intitulées protéines MDR faisant partie de la famille des transporteurs de type « ATP-Binding-Cassette » (**Vargas et al. 2014; Bugde et al. 2017**).

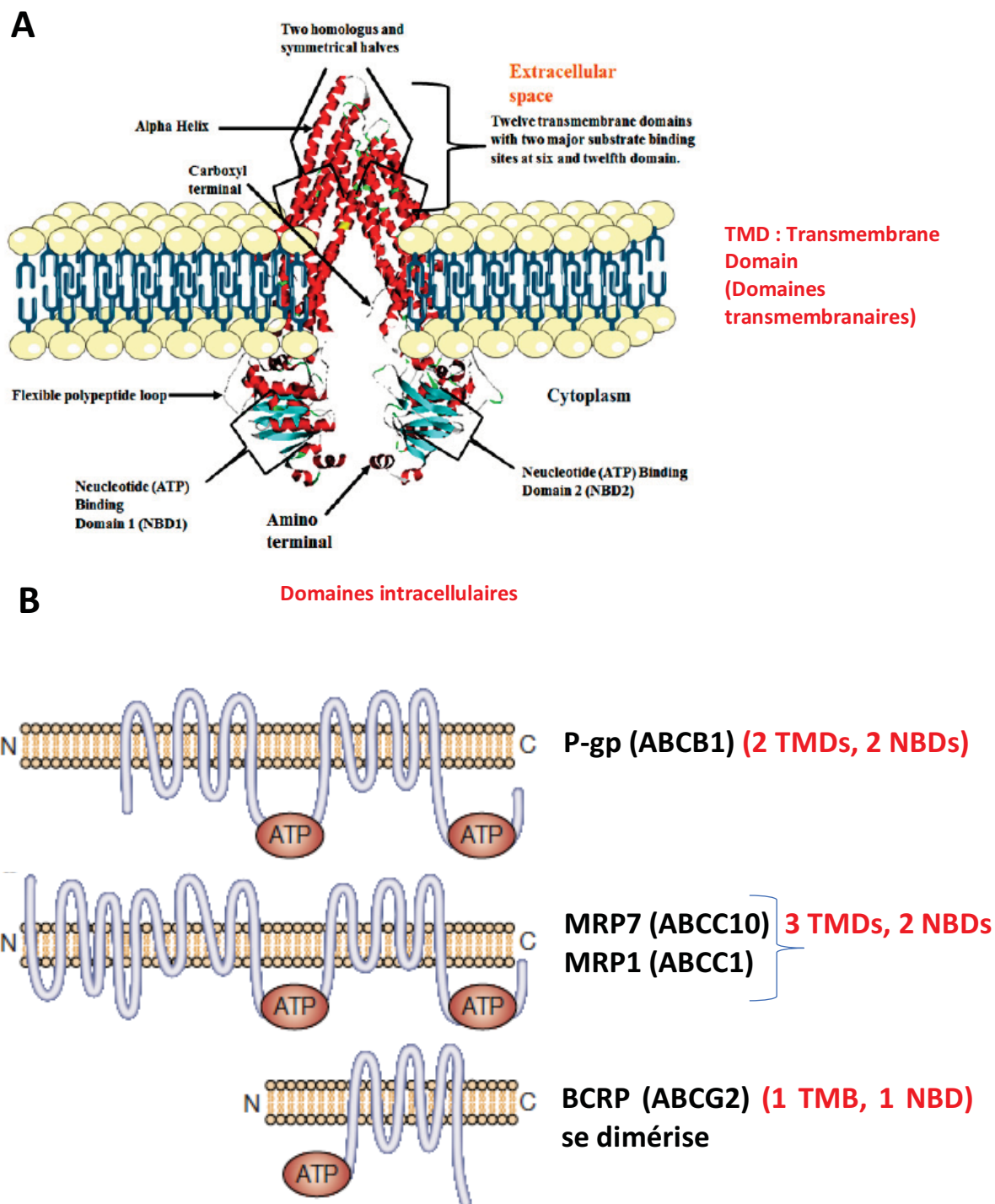


Figure 49: Structure de la P-gp (A) et des autres protéines de *MultiDrug Resistance* d'intérêt clinique majeur (B) (Gottesman, 2001, Dewanjee, 2017)

Table 1. ABC transporters and their expression in human tissues.

ABC transporter gene name	Protein name	Membrane localization	Tissue distribution	References
<i>ABCB1</i>	P-gp	Apical	Blood–brain barrier, brain parenchyma (astrocytes and microglia), placenta, liver, kidney, small intestine, blood–testis barrier, colon, and pancreas	[29,30]
<i>ABCC1</i>	MRP1	Basolateral	Blood–brain barrier, brain, breasts, colon, heart, kidney, lung, ovary, pancreas, placenta, prostate, skeletal muscle, skin, small intestine, spleen, and testis	[29,30]
<i>ABCC2</i>	MRP2	Apical	Blood–brain barrier, breasts kidney, liver, lung, pancreas, placenta, and small intestine	[29,30]
<i>ABCC3</i>	MRP3	Basolateral	Adrenals, colon, kidney, liver, liver, pancreas, placenta, prostate, small intestine, and spleen	[29,30]
<i>ABCC4</i>	MRP4	Apical, basolateral	Adrenals, blood–brain barrier, breasts, colon, heart, kidney, liver, lung, ovary, pancreas, placenta, prostate, skeletal muscle, skin, small intestine, smooth muscle, spleen, and testis	[29,30]
<i>ABCC5</i>	MRP5	Apical, basolateral	Blood–brain barrier, brain, heart, placenta, skeletal muscle, and smooth muscle	[29,30]
<i>ABCG2</i>	BCRP	Apical	Placenta, blood–brain barrier, seminal vesicle, liver, kidney, small intestine, and blood–testis barrier	[29,30]

Transporter tissue expression is not restricted to the ones indicated.

BCRP: breast cancer resistance protein.

Figure 50 : Localisation et fonctions des différents ABC transporteurs / MDR d'intérêt clinique (Bugde, 2017)

I) La Résistance tumorale médiée par les transporteurs « Multidrug Resistance »

La notion de « Multidrug Resistance » ou la résistance croisée des cellules cancéreuses vis-à-vis des agents anticancéreux est connue depuis longtemps. Elle se réfère principalement à une surexpression d'une série de transporteurs membranaires intitulés MDR. Ces protéines appartiennent à la super famille des transporteurs de type « ATP Binding Cassette » (ABC). Cette grande famille de protéines transmembranaires est composée de 48 gènes, subdivisés en 7 sous-familles classées de A à G, présentant une grande homologie de structure (**Bugde et al. 2017**). Les protéines **MDR** et plus généralement les transporteurs **ABC** sont identifiées à partir des séquences conservées spécifiques du domaine **ABC**. En général, ils sont composés (i) de domaines transmembranaires ou **TMD**, impliqués dans la reconnaissance et le transport des substrats et (ii) de domaines cytoplasmiques de liaison aux nucléotides ou **NBD**, constituant le site d'hydrolyse de l'ATP fournissant l'énergie nécessaire pour l'efflux des molécules (**Hollenstein, Dawson, et Locher 2007**). Les quatre principales classes de protéines MDR font partie des sous-familles **ABCB** (gène ABCB1 ou MDR1/ protéine P-glycoprotéine P-gp), **ABCC** (gène ABCC1/protéine « Multidrug Resistance protein 1 » MRP1 - gène ABCC2/ protéine « Multidrug Resistance protein 2 » MRP2 - gène ABCC10/ protéine « Multidrug Resistance protein 7 » MRP7), et **ABCG** (gène ABCG2/ « Multixenobiotic Resistance » MXR ou « Breast Cancer Resistance protein » BCRP) (**Sarkadi et al. 2006; Kathawala et al. 2014**) (**Figure 49**).

Ces transporteurs extrêmement conservés au cours de l'évolution sont très ubiquitaires. En effet, les protéines MDR sont exprimées à l'état naturel dans pratiquement tous les organismes vivants des procaryotes aux mammifères. Chez l'homme/les mammifères, plusieurs tissus normaux expriment le gène MDR1, telle que la barrière hématoencéphalique, la glande surrénale, le pancréas, le foie et le rein, les cellules hématopoïétiques et les cellules souches, entre autres (**Gottesman, Fojo, et Bates 2002**) (**Figure 50**). Chez les autres organismes, eucaryotes ou procaryotes (animal ou végétal), la présence d'**abcb1/mdr1/P-gp**, qui représente la pompe d'efflux membranaire de référence, a également été mise en évidence. En effet, à titre d'exemple, l'expression de l'ABCB1/MDR1/P-gp a été démontrée

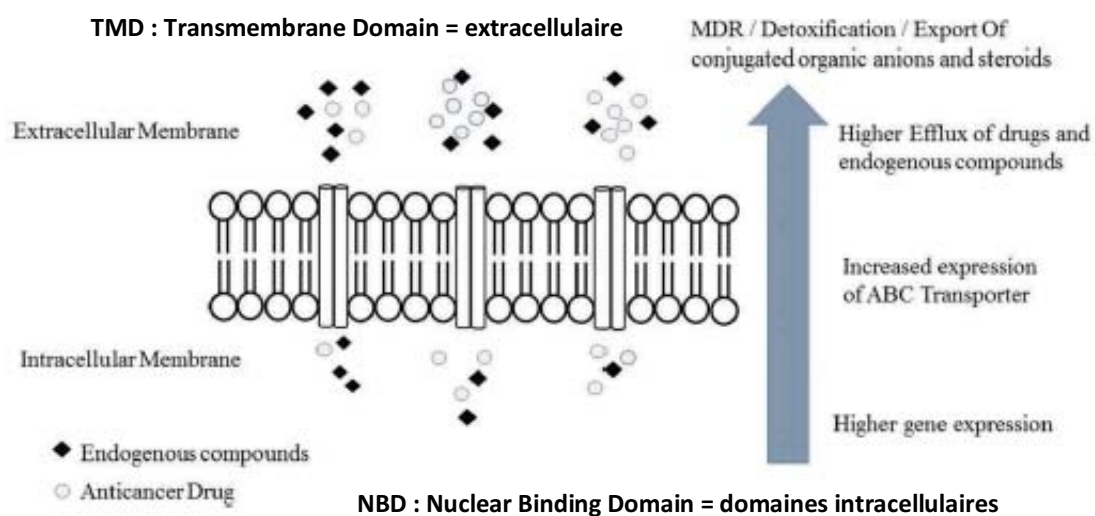


Figure 51 : Les protéines MDR ont un rôle d'efflux spécifique des xénobiotiques/médicaments du milieu intracellulaire vers le milieu extracellulaire (Bugde, 2017)

chez les bactéries et parasites (**Krogstad et al. 1992; Bottova et al. 2009**), chez les protozoaires (**Bamdad et al. 1997; Bamdad, Brousseau, et Denizau 1999**), chez la drosophile (**Vaché et al. 2006; Vache et al. 2007**) et chez le poisson (**Valton et al. 2013, 2015, 2017**).

Outre l'aspect structural et ubiquitaire, l'autre caractère commun des transporteurs MDR est leur fonction d'efflux de xénobiotiques/médicaments. En effet, il s'agit de transporteurs membranaires de type ATPasique avec un rôle détoxificateur. Ils expulsent vers le milieu extracellulaire les xénobiotiques/médicaments entrés passivement ou *via* d'autres transporteurs membranaires (**Figure 51**). Les pompes MDR reconnaissent des molécules de structures et de propriétés très variées (médicaments, polluants). Bien que les molécules prises en charge soient principalement des composés de type hydrophobe, les pompes MDR sont également capables d'expulser une grande variété de molécules amphipathiques* de type anions et/ou de cations. A titre d'exemple, la **P-gp** expulse préférentiellement les grosses molécules hydrophobes, tandis que les **MPRP** et **BCRP** peuvent transporter des médicaments hydrophobes et/ou de grands composés anioniques de type drogues conjugués (produits de biotransformation intracellulaire). Par ailleurs, chaque pompe serait capable de reconnaître et prendre en charge un panel varié de molécules chimiothérapeutiques et/ou de se relayer en réponse à la même chimiothérapeutique (**Dufour et al. 2015**) et/ou d'élargir son champ d'action par le mécanisme de chevauchements dans leur spectre reconnaissance de différentes molécules (**Hanahan et Weinberg 2000**). De part cette propriété, au sein de l'organisme, les transporteurs MDR jouent un véritable rôle de défense à l'encontre des xénobiotiques/médicaments à l'échelle cellulaire. De plus, leur implication est essentielle dans le maintien de l'homéostasie et le fonctionnement cellulaire normal. En effet, l'expression naturelle de ces protéines dans les tissus sains permet notamment de protéger les organes à risque tels que le cerveau, le tractus gastro-intestinal, le foie, le pancréas et le placenta, dans lesquels elles sont très fortement exprimées. En outre, 33% des cas de déficience dans les gènes des transporteurs **MDR** seraient associés à un tableau pathologique (**Dean 2005; Fukuda et Schuetz 2012**).

De par leur implication dans les mécanismes de détoxification cellulaire, les transporteurs **MDR** sont donc essentiels à la défense cellulaire contre des atteintes endogènes

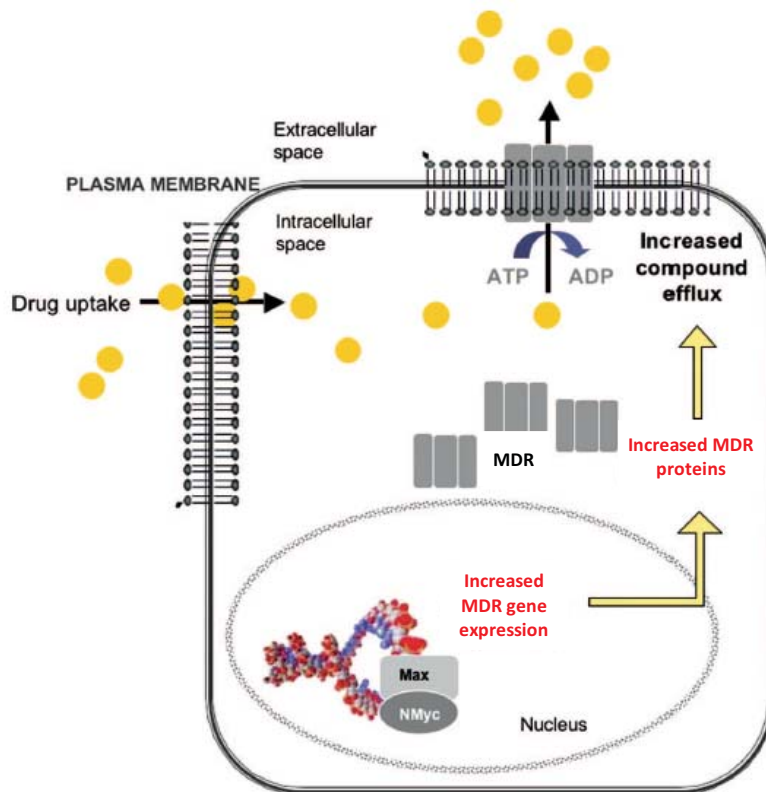


Figure 52 : L'augmentation de l'expression des protéines MDR est principalement expliquée par quatre mécanismes moléculaires : (1) une amplification, (2) une augmentation de la transcription, (3) des changements dans l'efficacité transcriptionnelle, (4) ou des réarrangements chromosomiques des gènes codant pour les pompes MDR (Munoz, 2007)

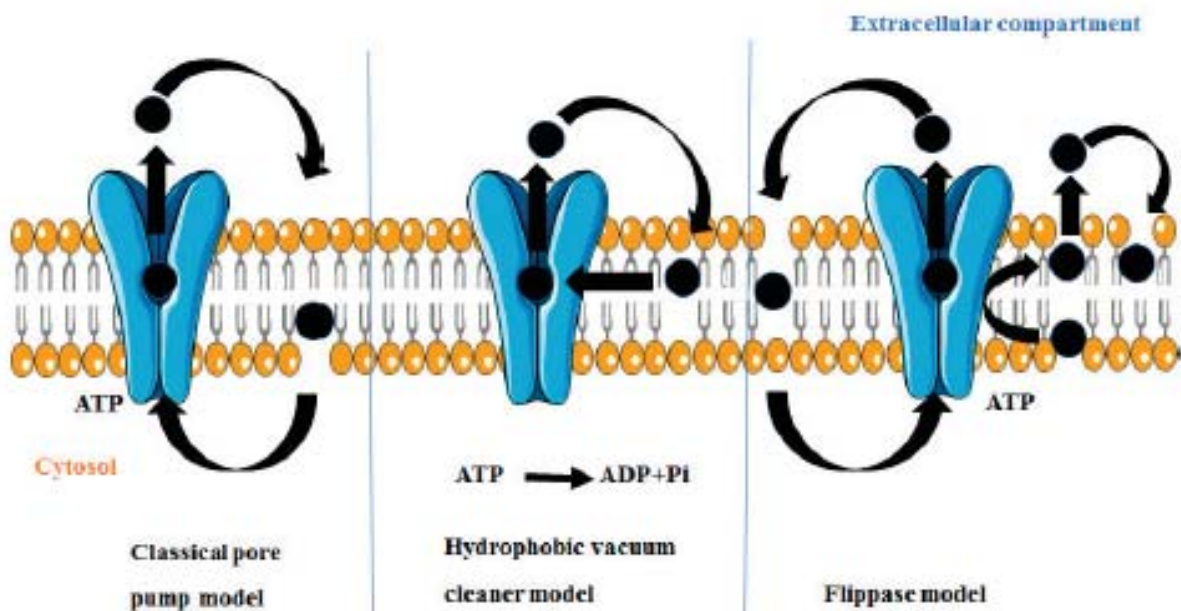


Figure 53 : Différents mécanismes d'efflux de la P-gp, (1) le modèle du pore classique, (2) le modèle de l'aspirateur hydrophobe, et (3) le modèle de *flippase** (Dewanjee, 2017)

et exogènes. Ainsi, les transporteurs MDR sont considérées comme un acteur majeur du concept de la « chimio-immunité » (Sarkadi et al. 2006; Vargas et al. 2014; Kathawala et al. 2014). Néanmoins, le mécanisme de ces pompes peut être détourné au profit de la survie des cellules tumorales. En effet, les pompes **MDR** sont fréquemment surexprimées chez les tumeurs. Cette surexpression est principalement expliquée par quatre mécanismes moléculaires : (1) une amplification, (2) une augmentation de la transcription, (3) des changements dans l'efficacité transcriptionnelle, (4) ou des réarrangements chromosomiques des gènes codant pour les pompes **MDR** (Bugde et al. 2017)(Figure 52). En outre, cette surexpression peut être impliquée dans la résistance innée (surexpression des pompes comme moteur de la cancérogénèse) ou acquise (surexpression suite aux traitements) vis à vis des molécules chimiothérapeutiques. De plus, le niveau d'expression de certaines pompes **MDR** majeures est positivement corrélé au degré d'indifférenciation et d'agressivité des tumeurs (Fletcher et al. 2010).

II) Structure et substrats des pompes MDR d'intérêt clinique

(a) La P-glycoprotéine

La p-glycoprotéine (P-gp) a été le premier transporteur de propriété MDR à être identifié, en 1976. Elle fait partie de la sous-famille **B** des transporteurs **ABC** et est codée par le gène **ABCB1** ou **MDR1**. Cette protéine dimérique de 170 kDa est composée de deux domaines transmembranaires (TMDs) et de deux domaines cytoplasmiques (NBDs) (Figure 49).

Plusieurs hypothèses sur le mécanisme d'efflux de la **P-gp** sont proposées dans la littérature : (1) un efflux ATP-dépendant simple, (2) un efflux par aspiration hydrophobe, et (3) un efflux modulé par un phénomène de *Flippase** (Shapiro et Ling 1997; Dewanjee et al. 2017) (Figure 53). Dans la première hypothèse, la P-gp génère un pore hydrophile dans la membrane lipidique, à travers lequel les molécules intracytoplasmiques sont éjectées. En outre, les principaux substrats de la P-gp étant majoritairement hydrophobes, ceux-ci se retrouvent généralement « coincés » entre le compartiment cytoplasmique aqueux et la

ABC transporters		Substrates
P-gp	ABCB1	Anticancer drugs: 5-fluorouracil, actinomycin D, bisantrene, chlorambucil, colchicine, cisplatin, cytarabine, daunorubicin, docetaxel, doxorubicin, epirubicin, etoposide, gefitinib, hydroxyurea, irinotecan (CPT-11), methotrexate, mitomycin C, mitoxantrone, paclitaxel, tamoxifen, teniposide, topotecan, vinblastine, and vincristine Antilipidemic: lovastatin and simvastatin Calcium channel blockers: azidopine, bepridil, diltiazem, felodipine, nifedipine, nisoldipine, nitrendipine, tiapamil, and verapamil Immunosuppressive agents: cyclosporine A, cyclosporine H, sirolimus, tacrolimus, and valspodar (PSC-833)
	ABCC1	Natural products: curcuminoids and flavonoids
MRP1	ABCC2	Daunorubicin, doxorubicin, imatinib, etoposide, teniposide, camptothecins, vinblastine, vincristine, and methotrexate
	ABCC3	Cisplatin, doxorubicin, epirubicin, etoposide, irinotecan, mitoxantrone, methotrexate, SN-38, vinblastine, vincristine, and oxaliplatin
BCRP	ABCC4	Methotrexate, etoposide, and teniposide
	ABCC5	Thiopurines, methotrexate, thioguanine, and topotecan
BCRP	ABCC6	6-mercaptopurine, methotrexate, gemcitabine, and zidovudine
	ABCC7	Anticancer drugs: daunorubicin, doxorubicin, epirubicin, etoposide, irinotecan, mitoxantrone, methotrexate, SN-38, topotecan, and bisantrene Antivirals: delavirdine, lopinavir, lamivudine, nelfinavir, and zidovudine

First Generation	Second Generation	Third Generation
Verapamil	(R)-Verapamil	Tariquidar (XR9576)
Cyclosporine A	Dexniguldipine	Zosuquidar (LY335979)
Vincristine	Elacridar (GF-120918)	Laniquidar (R101933)
Reserpine	Biricodar	ONT-093 (OC-144-093)
Quinidine	Dofequidar	Mitotane (NSC-38721)
Tamoxifen	Trifluoperazine	Annamycin
Trifluoperazine	Valspodar (PSC-833)	

Figure 54 : (A) Molécules prises en charge par la P-gp, MRP1 et BCRP (Bugde, 2017) et (B) les différentes générations d'inhibiteurs de P-gp développés (Dewanjee, 2017)

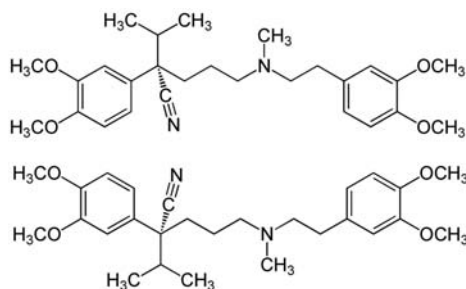


Figure 55: Structure du Vérapamil

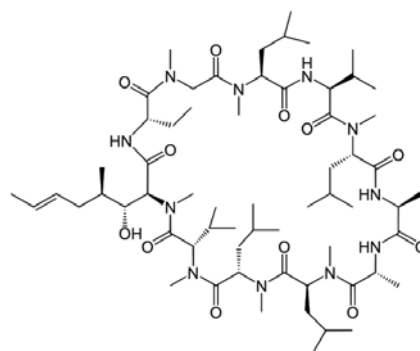


Figure 56 : Structure de la Cyclosporine A

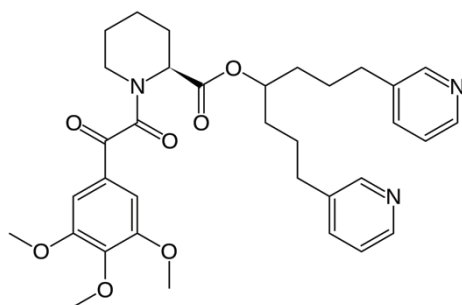


Figure 57: Structure du Biricodar

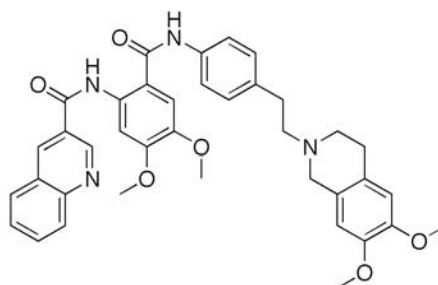


Figure 58 : Structure du Tariquidar

membrane lipophile. Le deuxième modèle proposé est intitulé « aspirateur hydrophobe ». Il implique une détection et un efflux des molécules pénétrant dans la cellule, et localisées dans la bicouche lipidique (couche interne). Ensuite, l'hydrolyse de l'ATP induit une éjection du composé dans le milieu extracellulaire. La troisième hypothèse, plus complète, implique un captage de la molécule alors située dans la couche interne de la membrane lipidique, suivi d'un phénomène de *Flippase*. Ce phénomène consiste au basculement des phospholipides d'un feuillet à l'autre de la bicouche lipidique, rendu possible par l'hydrolyse de l'ATP. Les molécules alors situées dans le compartiment interne se retrouvent en position externe, et sont ensuite éjectées dans le milieu extracellulaire (**Higgins et Gottesman 1992**).

La **P-gp** est principalement à l'origine de l'efflux d'une large gamme de xénobiotiques notamment les grosses molécules hydrophobes, présentant des tailles de 300 Da à 4000 Da. Les molécules cibles de la P-gp dans les tumeurs sont principalement des taxanes, des vinca-alcaloïdes, des anthracyclines, des Inhibiteurs de Tyrosines Kinases (ITK), des camptothécines et les inhibiteurs de PARP1, entre autres (**Figure 54**) (**Dewanjee et al. 2017; Bugde et al. 2017; Dufour et al. 2015; Han, Gao, et Mao 2018, 1**).

Une des stratégies développées pour lutter contre la chimiorésistance induite par la **P-gp** a été l'usage d'**inhibiteurs spécifiques** (**Figure 54**). Ceux-ci ont pour objectif **(1)** d'altérer l'activité ATPasique de la **P-gp**, et/ou **(2)** d'altérer son expression, et/ou **(3)** de générer une compétition préférentielle pour l'efflux de la **P-gp**. Dans cette optique, des inhibiteurs de **P-gp** de première génération (vérapamil, un bloqueur des canaux calciques, cyclosporine A, un immunosuppresseur, peu spécifiques) (**Figures 55 et 56**), de deuxième (Bircodan, un dérivé du Tacrolimus, assez spécifique) (**Figure 57**), et de troisième génération (Tariquidar, très spécifique) (**Figure 58**), ont été développés depuis une vingtaine d'années. Ceux-ci ont montré des résultats plus ou moins concluants dans les tests précliniques et cliniques en combinaison avec des molécules chimiothérapeutiques, mais aucun n'a été retenu à ce jour. En effet, la **P-gp** étant surexprimée dans un nombre important de tissus, le principal obstacle à l'utilisation de ces inhibiteurs réside dans leur forte toxicité secondaire, ainsi que leur manque de spécificité (possible inhibition croisée d'autres transporteurs **ABC**). De plus, un certain nombre de ces inhibiteurs (surtout ceux de première génération) sont détournés de leur propriété

pharmacologique initiale, induisant des toxicités secondaires sur les organes cibles. En effet, le vérapamil qui est un régulateur du rythme cardiaque, génère des effets secondaires notoires sur le système cardiovasculaire. Un autre inconvénient majeur de ces inhibiteurs est leur potentielle altération du cytochrome p450, nécessaire au métabolisme des molécules anticancéreuses. Alors utilisés en co-traitement avec ces médicaments, les inhibiteurs de P-gp peuvent changer la pharmacocinétique du composé et le rendre plus toxique, ou moins efficace (**Dewanjee et al. 2017**). Ainsi, les stratégies de lutte contre la chimiorésistance induite par des inhibiteurs sont très complexes.

Parallèlement, d'autres stratégies ont été envisagées, telles que **(1)** le développement de nouvelles molécules n'étant pas substrats des pompes, ou **(2)** le développement de molécules de transport modifiant le mécanisme de pénétration intracellulaire des médicaments (**Vargas et al. 2014; Westover et Li 2015**). Toutes ces stratégies sont pour l'instant à l'état de développement préclinique.

(b)La protéine MRP1

La pompe de Multidrug-Resistance Protein 1 (MRP1), codée par le gène ABCC1, est une protéine multimérique de 190 kDa (**Cole 2014**). Elle est constituée de trois domaines transmembranaires (TMD) et deux domaines cytoplasmiques (Figure) (**Lu, Pokharel, et Bebawy 2015**)(Figure 49). Cette pompe reconnaît et expulse une variété de grosses molécules hydrophobes et notamment des molécules chimiothérapeutiques et/ou de grands composés anioniques de type drogues conjugués (produits de biotransformation intracellulaire). Parmi les substrats connus, on retrouve des antimétabolites, des anthracyclines, des vinca-alcaloïdes mais également des thérapies antihormones (Figure 54). La protéine MRP1 est impliquée dans la résistance aux traitements chez les cancers du poumon, de la prostate et du sein. Notamment, son expression augmenterait en fonction du stade et du caractère invasif des tumeurs prostatiques (**Munoz et al. 2007**). De plus, elle serait fortement exprimée dans les métastases ganglionnaires des cancers du sein, suggérant une implication potentielle de cette

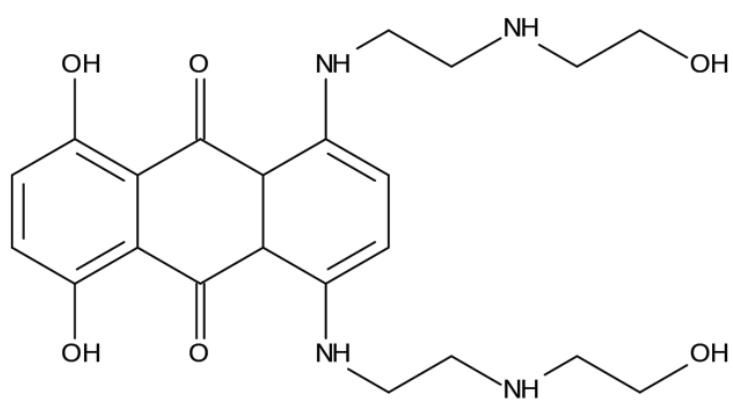


Figure 59: Structure du Mitoxantrone

pompe dans la progression de la maladie au stade métastatique (**Munoz et al. 2007, 1; Lu, Pokharel, et Bebawy 2015, 1; Cole 2014, 1**).

(c) La protéine MRP7

La Multidrug-Resistance Protein 7 (**MRP7**), codée par le gène **ABCC10**, est le dixième membre de la superfamille **C** des transporteurs **ABC**. Cette protéine, récemment identifiée comme ayant des propriétés « Multidrug Resistance » un poids moléculaire de 171 kDa. Elle est composée de trois domaines **transmembranaires** et deux domaines cytoplasmiques (**Figure 49**). Peu de données existe à l'heure actuelle, sur les propriétés de la MRP7. Elle reconnaît un large panel de molécules antinéoplasiques telles que les taxanes, l'épothilone B, les vinca alcaloïdes, la cytarabine ainsi que les modulateurs de la voie oestrogénique tel que le tamoxifène (**Kathawala et al. 2014; Chen et al. 2003, 10; Anreddy et al. 2014; Domanitskaya et al. 2014**).

(d) La protéine BCRP

La pompe "Breast Cancer Resistance Protein" ou BCRP, codée par le gène **ABCG2**, est une protéine monomérique de 72 kDa composée d'un seul domaine transmembranaire et d'un domaine cytoplasmique (**Hira et Terada 2018; Brackman et Giacomini 2018; Westover et Li 2015**)(**Figure 49**). En outre, son action d'efflux transmembranaire est possible suite à la dimérisation de deux protéines **BCRP**. La **BCRP** a initialement été découverte sur des modèles de tumeurs mammaires résistantes à diverses molécules chimiothérapeutiques telles que le cisplatine, le paclitaxel, la vinblastine ou encore le mitoxantrone (**Figure 59**), d'où son appellation « Breast Cancer Resistance ». Chez l'Homme, la **BCRP** est également exprimée dans tissus et/ou organes sains comme le tractus gastro-intestinal, le foie, les reins, le système nerveux central, le tissu mammaire, ovarien, et testiculaire, les glandes surrénales et le placenta (**Hira et Terada 2018**). La BCRP est également fortement exprimée dans les tumeurs et elle est à l'origine d'une chimiorésistance. Les substrats de la BCRP constituent principalement des médicaments hydrophobes, des cations organiques, des acides organiques ou de grands composés anioniques de type drogues conjuguées (produits de

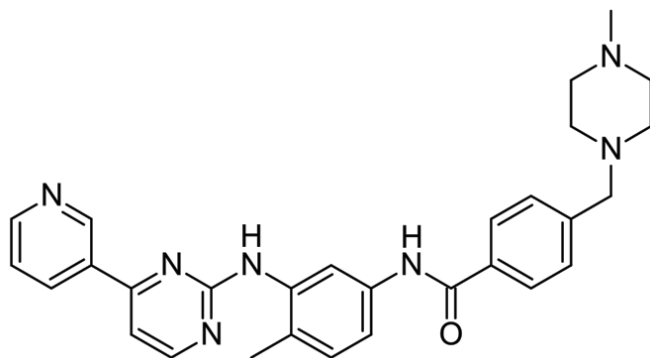


Figure 60: Structure de l'Imatinib

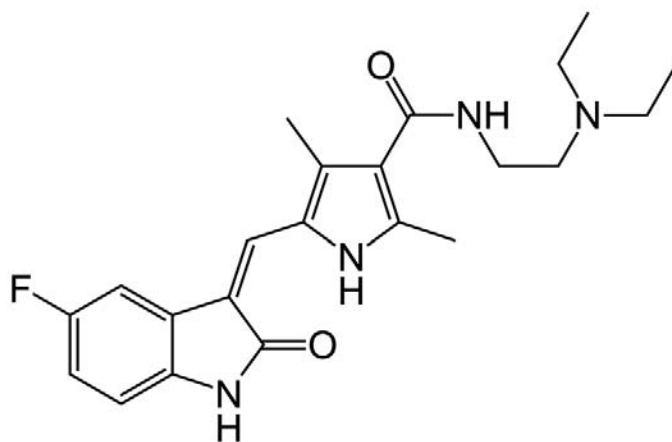


Figure 61: Structure du Sunitinib

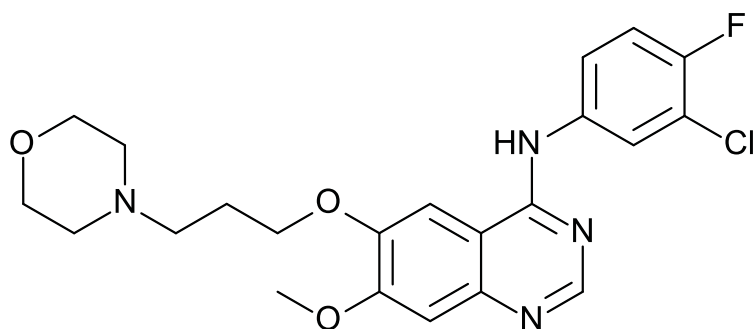


Figure 62: Structure du G  fitinib

biotransformation intracellulaire). Outre de nombreuses molécules chimiothérapeutiques, plusieurs **ITK** oraux, tels que l'imatinib, le sunitinib et le géfitinib (**Figures 60, 61 et 62**) ont également été rapportés comme étant les substrats de la **BRCP**. De plus, certains substrats de la **BRCP** se chevauchent avec ceux de la **P-gp**.

L'ensemble de ces pompes peuvent être surexprimées chez les tumeurs de manière séquentielle ou simultanée, afin de potentialiser les capacités d'efflux des molécules chimiothérapeutiques. A titre d'exemple, au sein de notre équipe de recherche, une expression de type-relai de la **P-gp** et de la **BCRP** a été mise en évidence sur des modèles de tumeurs mammaires TN en conditions *in vitro* en présence de l'Olaparib, un inhibiteur de PARP1, après 3 et 6h de traitement (**Dufour et al. 2015**). Ainsi, l'étude du niveau d'expression et de la cinétique d'expression de ces pompes au cours des traitements et à long terme permet de mieux appréhender la sensibilité des tumeurs vis-à-vis des traitements.

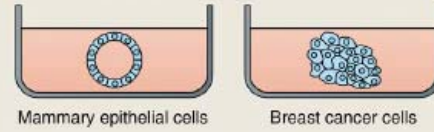
E) La culture cellulaire en trois dimensions

Le choix et le développement de modèles pour les études précliniques est une étape primordiale pour la sélection et la caractérisation des molécules thérapeutiques d'intérêt, en termes d'efficacité biologique et de toxicité. Les méthodes de culture cellulaire traditionnelles en monocouches (2D) ont été utilisées depuis des dizaines d'années afin de définir l'efficacité biologique des composés thérapeutiques. A titre d'exemple, elles ont permis de sélectionner et d'identifier des molécules d'intérêts afin de lutter contre des grandes maladies telles que le Parkinson, le SIDA, le diabète ou encore le cancer (**Antoni et al. 2015; Breslin et O'Driscoll 2016; Costa et al. 2016**). Les cultures cellulaires en 2D présentent l'avantage d'être rapides, peu coûteuses et très reproductibles (**Antoni et al. 2015**). Cependant ces modèles ne reflètent pas les échanges cellulaires, ni le microenvironnement ni les caractéristiques morphologiques et fonctionnelles du tissu originel (**Antoni et al. 2015**).



Figure 63 : Photo du professeur N. Bissell, considérée comme la mère de la culture 3D à partir du tissu mammaire, dans son laboratoire en 1974 (Bissell, 2017)

1989 Barcellos-Hoff et al. 1992 Petersen et al.
Use of a laminin-rich matrix to develop assays of mammary morphogenesis and to distinguish between healthy and malignant human epithelial cells.



2001 Simian et al.
Use of 3D collagen cultures to study the mechanisms of mammary gland branching morphogenesis

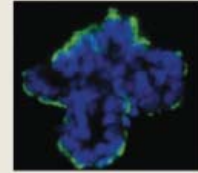


Figure 64 : Premières expériences de cultures 3D de tissus mammaires en présence de gels riches en laminine. Génération de structures saines ressemblant à des acini, se distinguant des structures cancéreuses. Premières études sur la morphogénèse de la glande mammaire à partir des cultures 3D (Simian and Bissell 2017)

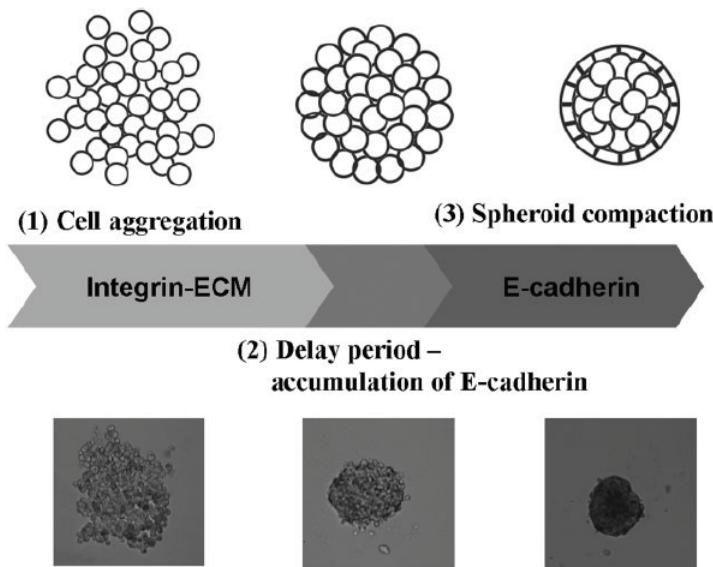


Figure 65 : Schéma de formation des sphéroïdes, divisé en trois étapes : (1) formation d'agrégats cellulaires lâches via la création de liaisons intégrines/matrice, (2) expression et accumulation de l'E-cadhérine, (3) formation de sphéroïdes compacts par la création d'interactions cadhérines/cadhérines (Lin, 2008)

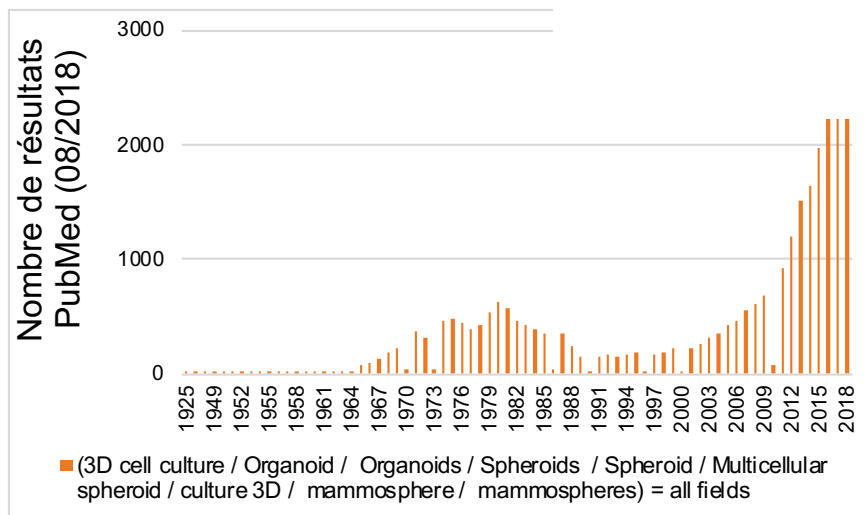


Figure 66 : Nombre d'occurrences des publications sur la culture 3D répertoriées depuis 1925 à nos jours (données extraites de PubMed)

Dans ce contexte, la découverte et l'isolation des protéines matricielles ont permis de définir de nouvelles perspectives d'évolution de la culture cellulaire *in vitro* traditionnelle. En effet, depuis une trentaine d'années, le rôle primordial du microenvironnement et notamment de la matrice extracellulaire a été mis en évidence pour leur impact sur la détermination du destin cellulaire. A la même période, les études du Professeur N. Bissell (**Figure 63**) mettaient en évidence un retour à un état cellulaire fonctionnel proche du tissu d'origine, chez des cellulaires mammaires saines cultivées au sein de gels contenant des protéines matricielles (**Marina Simian et Bissell 2017; Bissell 2017; Inman et al. 2015**). Notamment, les cellules mammaires récupéraient (1) leur structure tridimensionnelle d'origine sous forme d'acini, et (2) leur capacité à produire du lait (**Furuta et Bissell 2016; Inman et al. 2015; Barcellos-Hoff et al. 1989; Xiuli Wang et Kaplan 2012**). Par ailleurs, ces types de cultures permettaient d'identifier les tissus sains des tissus tumoraux (**Figure 64**).

La culture cellulaire en 3D* consiste ainsi en la formation d'amas cellulaires compacts et organisés souvent de forme ovoïde, formés dans une matrice extracellulaire propice à la génération de jonctions et communications intercellulaires (**Figure 65**). Ces amas vont former un micro-tissu organisé, composé de plusieurs couches cellulaires permettant de reconstituer l'hétérogénéité tissulaire *in vivo* (**Marina Simian et Bissell 2017**). Les cultures 3D peuvent être composées d'un ou plusieurs types cellulaires (modèles de mono ou de co-cultures*) comprenant des cultures primaires (issues de fragments tissulaires), des cellules souches, des cellules issues de lignées immortalisées ou de lignées tumorales. En outre, selon le type cellulaire utilisé, la culture 3D n'aura pas la même appellation : on parlera d'**organoïdes*** pour des cultures dérivées de cellules souches ou d'explants tissulaires, et plutôt de **sphéroïdes*** ou de **Multi Cellular Spheroids (MCS)** pour des mono ou co-cultures* de cellules tumorales et/ou immortalisées (**Marina Simian et Bissell 2017**). Pour les cas particuliers des cultures de cellules mammaires, les cultures 3D s'intitulent *mammosphères* (**Costa et al. 2016**). Depuis une vingtaine d'années, le développement des cultures est en plein essor (**Figure 66**).

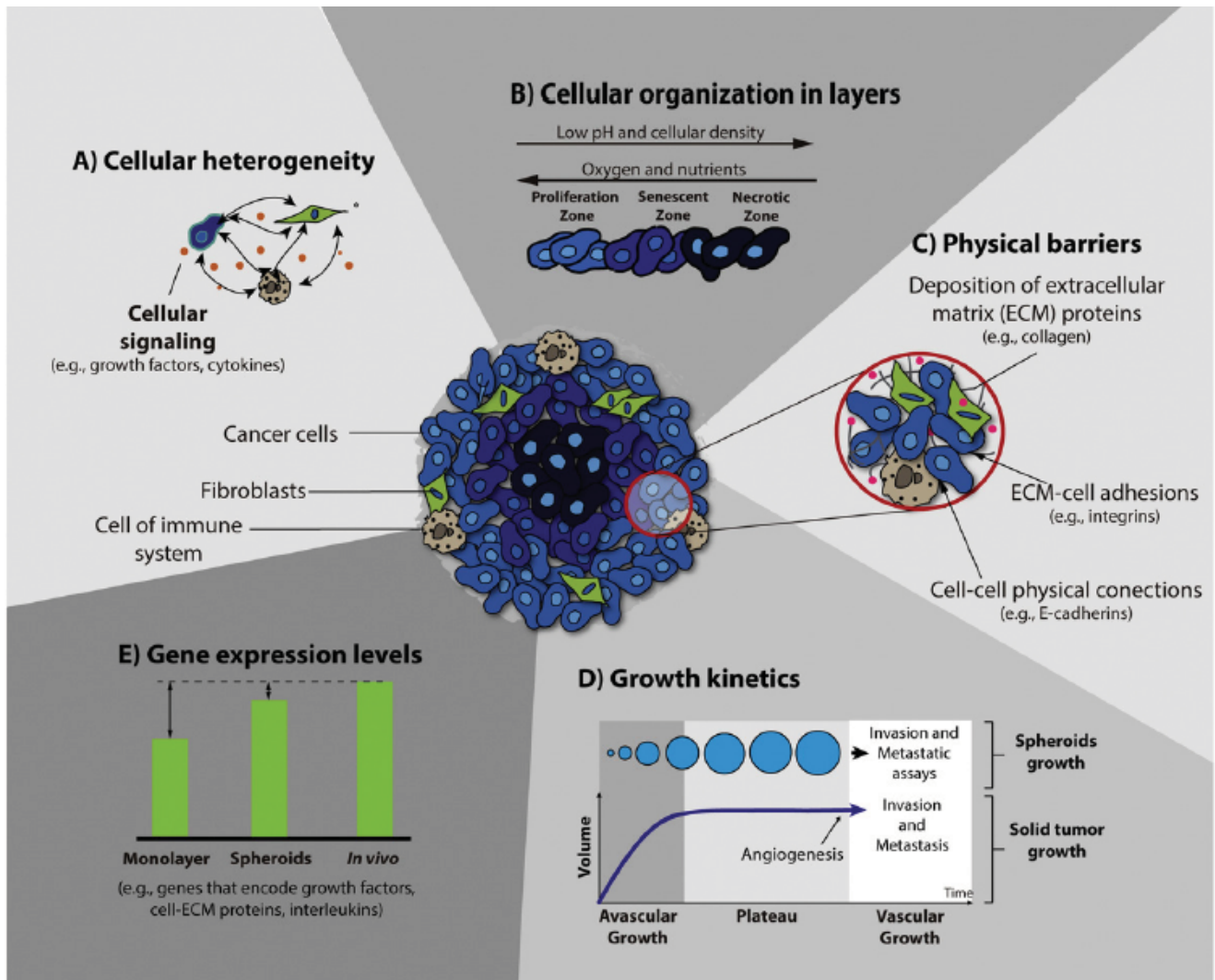


Figure 67 : Représentation schématique des caractéristiques principales des cultures 3D, permettant de confirmer leur biomimétisme vis-à-vis des tumeurs *in vivo* (Costa, 2016)

I) Caractéristiques et intérêts des cultures cellulaires « 3D »

Les cultures cellulaires 3D permettent de mimer le microenvironnement* tissulaire normal ou tumoral. Plus spécifiquement dans le domaine de l'Oncologie, les sphéroïdes représentent un modèle de choix pour étudier l'efficacité des thérapies antinéoplasiques. En effet elles permettent de recréer les caractéristiques principales des tumeurs *in vivo* par (1) les interactions cellules/matrice et cellules/cellules, (2) la cinétique de croissance, (3) l'hétérogénéité cellulaire et les gradients métaboliques, (4) le profil d'expression génique et enfin (5) la résistance aux médicaments (Costa et al. 2016) (Figure 67).

(a) Interactions cellules/matrice et cellules/cellules

Les phénomènes d'adhésion et de différenciation contribuent à la formation de sphéroïdes. En outre, celle-ci s'effectue en deux étapes : (i) la phase d'agrégation cellulaire qui est réalisée soit par la gravité, soit par des forces mécaniques (cf. *méthodes de formation des sphéroïdes*) induisant ainsi une *up-regulation* des molécules impliquées dans l'adhésion intercellulaire (les cadhérines et intégrines) suivie d'une phase (ii) de liaison physique cellules/cellules et cellules/matrice extracellulaire suite au rajout de celle-ci dans les cultures (Lin, Lin, et Chang 2008)(Figure 65 et 67). Par ailleurs, certains types cellulaires sont capables de produire leur propre matrice afin de consolider ces liaisons, et de la déposer à la surface des micro-tumeurs.

(b) Cinétique de croissance des tumeurs avasculaires

Le développement tumoral se décompose en trois phases : (1) une phase de croissance exponentielle avasculaire, (2) une phase de latence, préalable à la formation de néo vaisseaux permettant une oxygénation des zones profondes, (3) une phase vasculaire de reprise de la croissance tumorale suivie d'une phase d'invasion. La phase de latence est notamment activée par un excès de cellules hypoxiques centrales. Ces dernières produisent des facteurs pro-angiogéniques induisant la formation de néo-vaisseaux et des métalloprotéases capables de

Spheroid Characteristics

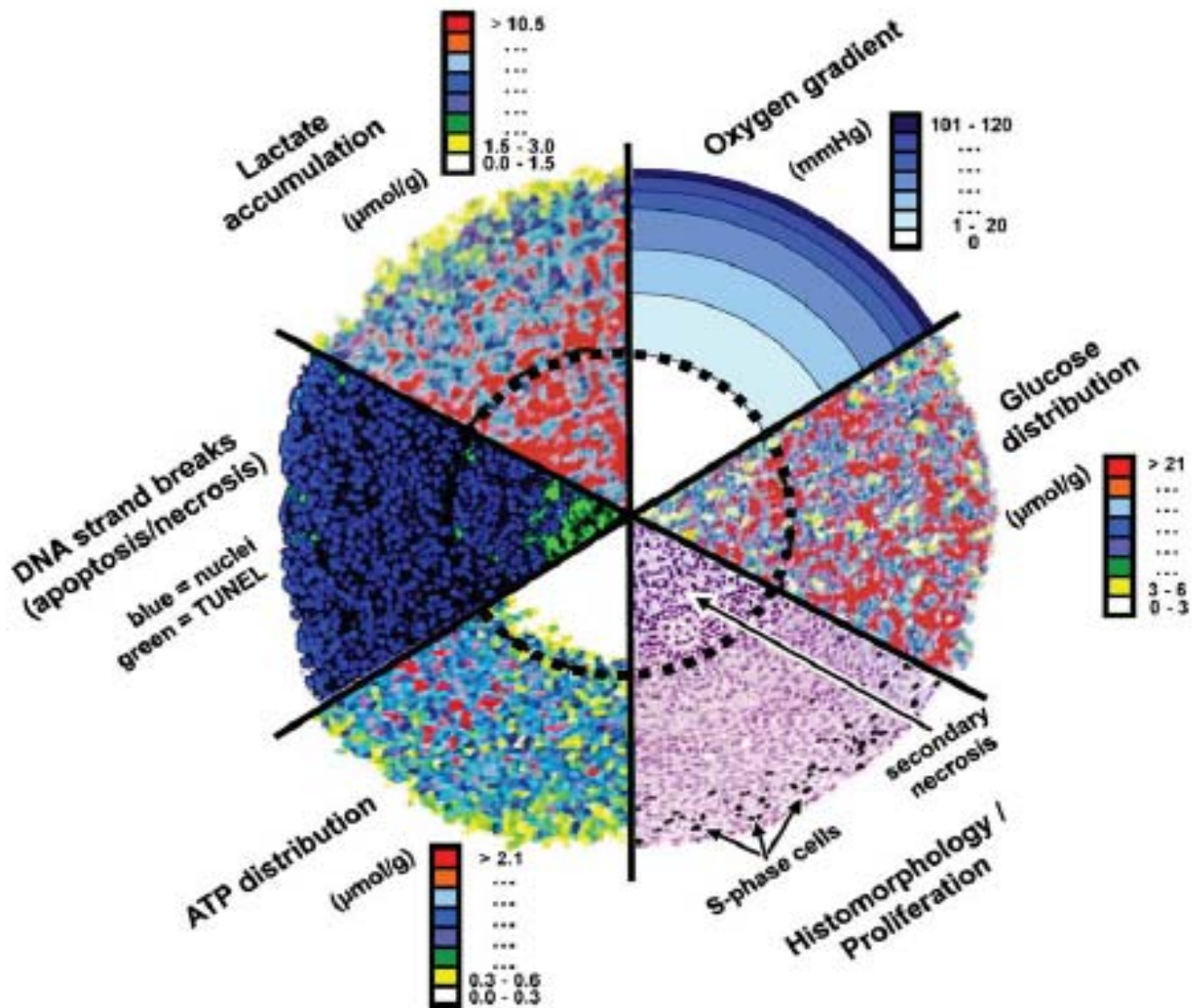


Figure 68 : Représentation schématique des différents gradients biochimiques et cellulaires retrouvés dans les modèles de cultures en 3D (Hirschhaeuser, 2010)

dégrader la matrice. En outre, les modèles de sphéroïdes en monoculture présentent généralement une phase de croissance exponentielle (jusqu'à 500 à 1000 μm de diamètre), suivie d'une phase de latence (phase plateau), permettant de mimer la cinétique de croissance des tumeurs avasculaires (**Figure 67**). Par ailleurs, certains modèles de co-cultures avec des cellules endothéliales permettent de modéliser les phases de croissance vasculaires, bien que ces techniques soient difficiles à mettre en place. En effet, les méthodes de culture nécessitent une optimisation fine permettant de maîtriser le ratio de prolifération des différents types cellulaires contenus dans le sphéroïde (**Costa et al. 2016; Duval et al. 2017**).

(c) Hétérogénéité cellulaire et des gradients métaboliques

Les sphéroïdes *in vitro* développent un microenvironnement hétérogène similaire aux tumeurs avasculaires *in vivo*. En effet, au fur et à mesure de leur croissance et selon l'âge des sphéroïdes, des gradients d'accès à (i) l'oxygène (de la périphérie vers l'intérieur), (ii) aux nutriments (de la périphérie vers l'intérieur) ainsi (iii) qu'une accumulation des déchets du métabolisme cellulaire sont détectés au sein des sphéroïdes. Les cellules situées au centre des sphéroïdes en conditions hypoxiques* maintiennent leur survie en activant leur métabolisme anaérobie *via* l'effet Warburg, par la production de lactate, dont l'accumulation est toxique pour les cellules (**Trédan et al. 2007**)(**Figure 68**). De plus, le transport des nutriments et des métabolites étant limitées pour ces cellules internes, une augmentation du nombre de cassures double-brin est détectée à leur niveau. Pour les cellules des feuilletts intermédiaires, avec un accès moins limité aux nutriments et à l'oxygène, un état de quiescence ou de sénescence est fréquemment retrouvé. En revanche, les cellules situées en périphérie ont un accès optimal aux nutriments et à l'oxygène. Elles ont alors la capacité de proliférer dans ces conditions. Ainsi, il a été démontré que des sphéroïdes présentant des tailles supérieures à 500 μm contenaient (1) un noyau de cellules hypoxiques ou nécrotiques, entouré de (2) cellules quiescentes ou sénescents, et (3) d'un anneau de cellules métaboliquement actives / proliférantes (**Figure 67**) (**Hirschhaeuser et al. 2010; Costa et al. 2016**).

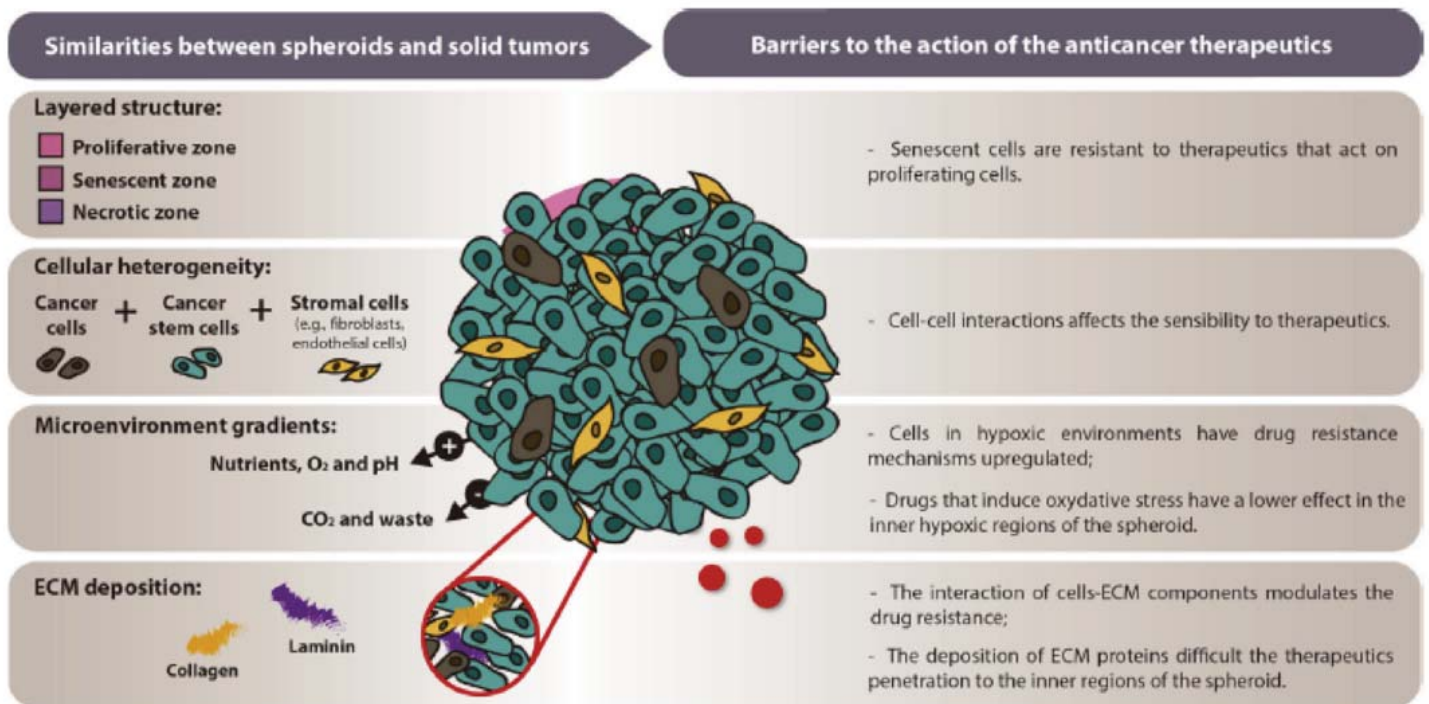


Figure 69 : Intérêt des caractéristiques cellulaires et du microenvironnement des modèles de cultures cellulaires en 3D pour l'étude de la sensibilité et de la résistance des molécules anticancéreuses (Costa, 2017)

(d) Profil d'expression génique

L'ensemble de cette hétérogénéité cellulaire et métabolique retrouvée au sein des sphéroïde implique la formation d'une hétérogénéité moléculaire. Cette propriété a été mise en évidence chez différents modèles de sphéroïde, par l'analyse de l'expression de gènes codants pour des facteurs de croissance, des facteurs pro-angiogéniques et des molécules d'adhésions. Le profil d'expression génique des cultures 3D s'est donc avéré plus proche des tumeurs *in vivo* que celui de cellules cultivées en monocouche (**Figure 67**) (**Costa et al. 2016**).

(e) Résistance aux médicaments

L'ensemble des jonctions intercellulaires générées lors de la formation des sphéroïdes forme une barrière physique naturelle pour la pénétration et la distribution des agents thérapeutiques au sein des sphéroïdes, également appelé transport de masse limité (Trédan et al. 2007). De plus, les interactions cellulaires au sein des sphéroïdes augmentent la densité de ces micro-masses, induisant une pression des fluides interstitiels accrue (IFP pour *Interstitial Fluid Pressure*). Cette dernière peut également générer un défaut de pénétration des médicaments dans ces micro-masses (**Minchinton et Tannock 2006**). Par ailleurs, la présence des gradients d'accès à l'oxygène au sein des sphéroïdes génère un environnement hypoxique central, capable de modifier la charge des molécules thérapeutiques. Ceci peut changer les propriétés physicochimiques des molécules, les rendant potentiellement inefficaces, ou limitant leur capacité de pénétration intracellulaire. Finalement, la présence de cellules sénescents ou quiescentes dans les zones intermédiaires de ces micro-tumeurs permettent de modéliser l'hétérogénéité la faible efficacité des molécules thérapeutiques agissant sur des cellules en division.

L'ensemble de ces caractéristiques permettent de confirmer l'intérêt des modèles de culture 3D pour l'étude de l'efficacité et de la résistance des molécules antinéoplasiques (**Costa et al. 2014**) (**Figure 69**).

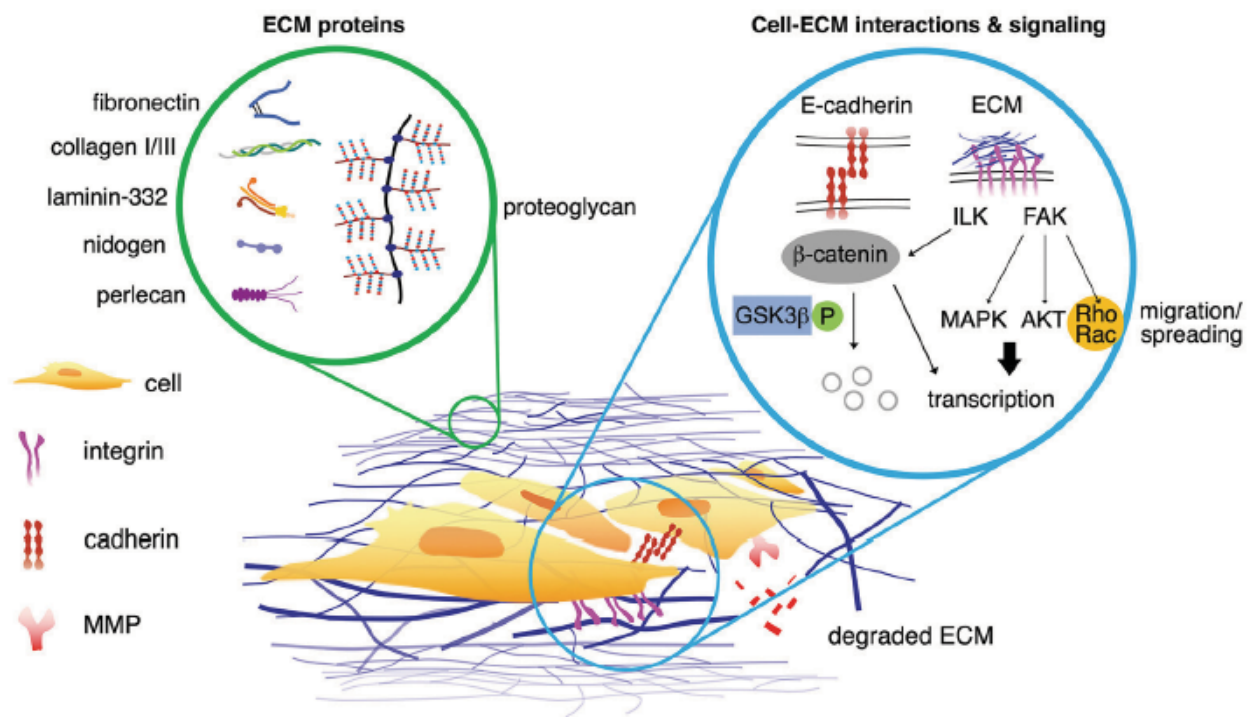


Figure 70 : La matrice extracellulaire est généralement composée d'un réseau complexe de molécules, interagissant entre elles et avec des récepteurs et protéines cellulaires, permettant la formation de microtissus, ainsi que la différenciation et la prolifération cellulaire. Ces interactions, extrêmement régulées, peuvent être modifiées au profit des cellules cancéreuses, afin de permettre leur échappement et l'invasion métastatique (Belgodere, 2018)

- Abundant components
 - Laminin
 - Type IV collagen
 - Perlecan (heparan sulfate proteoglycan)
 - Nidogen/entactin
- Proteases
 - 72 kda MMP-2
 - 92 kda MMP-9
 - urUokinase
 - Tissue type plasminogen activator
- Growth factors
 - TGF β s
 - FGF
 - EGF
 - PDGF
 - IGF
- Other proteins
 - Amylase
 - Transferrin
 - Clusterin

Figure 71 : Principaux composants de la matrice extracellulaire (Kleinman 2005). La plupart sont retrouvés dans les solutions commerciales telles que le Matrigel® ou le Geltrex® à des concentrations physiologiques

(f) Technologies de formation des cultures cellulaires 3D

Un très grand nombre de technologies de formation des cultures cellulaires 3D ont été décrites dans la littérature. Le principal objectif de l'ensemble de ces technologies est de favoriser les contacts intercellulaires et cellules/matrice au détriment des contacts cellules/support de culture (**Lin, Lin, et Chang 2008**). Les deux principales méthodologies décrites permettant la formation des sphéroïdes sont (i) celles utilisant des **échafaudages cellulaires** appelée technique en « *scaffold** » et (ii) celles ne nécessitant pas d'échafaudages, appelée « *scaffold-free** ». Quelle que soit la méthode utilisée, le recours à l'ajout de protéines matricielles (Matrigel®*) ou autres, est indispensable pour la création de microstructures biomimétiques.

❖ Rôle de la matrice extracellulaire dans la formation des sphéroïdes

La matrice extracellulaire permet de maintenir l'intégrité tissulaire et de séparer les différents compartiments. De plus, elle présente des fonctions de barrière physique entre les cellules et les molécules, de traduction des signaux mécaniques et d'autres fonctions biologiques nécessaires au maintien de la spécificité tissulaire (Benton et al. 2014). Dans les années 1980, les premières protéines matricielles ont été isolées, produites à partir de cellules de chondrosarcome de souris (cellules **EHS** du nom de leurs découvreurs, Engelbreth, Holm and Swarm). Celles-ci formaient un gel dense composé de laminine 11, de collagène IV, d'héparan sulfate proteoglycan et d'un certain nombre de facteurs de croissance (**FGF, EGF, IGF, TGFb**) (**Kleinman et Martin 2005; Benton et al. 2014**)(**Figure 70 et 71**). Ce gel, reproduisant la composition de la membrane des cellules de mammifères, a permis de promouvoir la croissance et la différenciation d'un grand nombre de lignées cellulaires, de cellules primaires et également d'explants tissulaire (**Swarm 1963; Orkin et al. 1977; Kleinman et Martin 2005; Benton et al. 2014**). Suite à ces travaux, des études utilisant des modèles d'explants tumoraux ou de lignées cellulaires cultivées sur ces matrices ont permis de mimer plus fidèlement le microenvironnement des tumeurs (**Tauro et Lynch 2018; Belgodere et al. 2018; M. Simian et al. 2001**). De plus, ces microstructures permettaient de

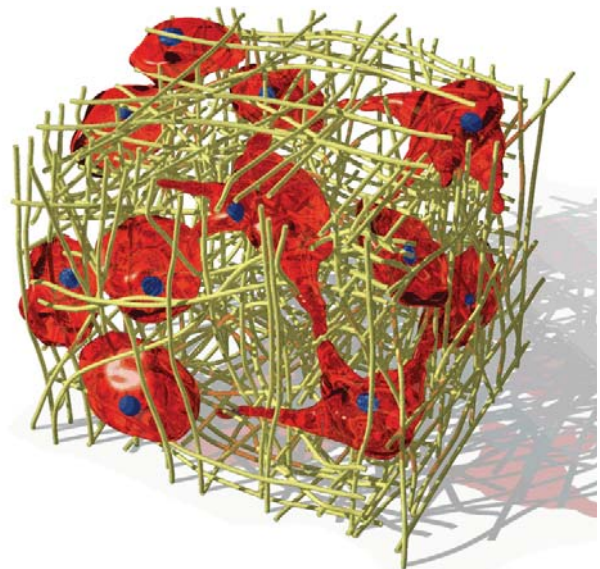
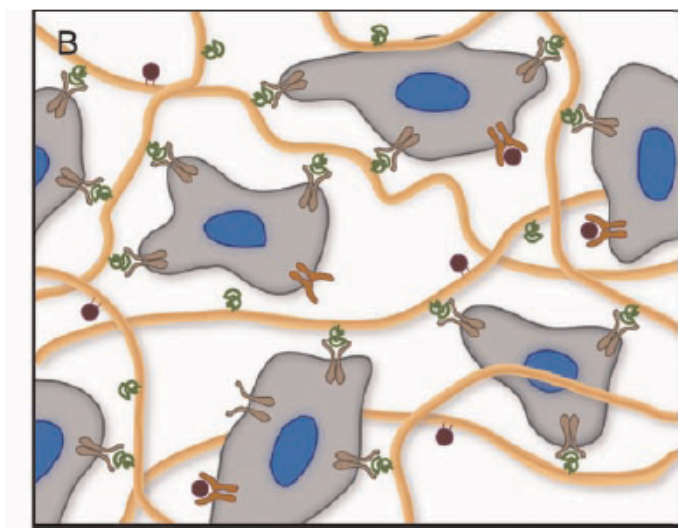


Figure 72 : Représentation des hydrogels utilisés dans les techniques de formation des sphéroïdes par *scaffold*. Les cellules sontensemencées au sein de ces gels, déposés dans des microplaques de culture. Celles-ci vont petit à petit former des jonctions avec les intégrines présentes sur les bio polymères (protéines en vert, figure de gauche), et ensuite se rejoindre pour former un microtissu organisé et compact (vue 2D et 3D) (Tibbitt, 2009 et <https://goo.gl/images/NL6nz9>)

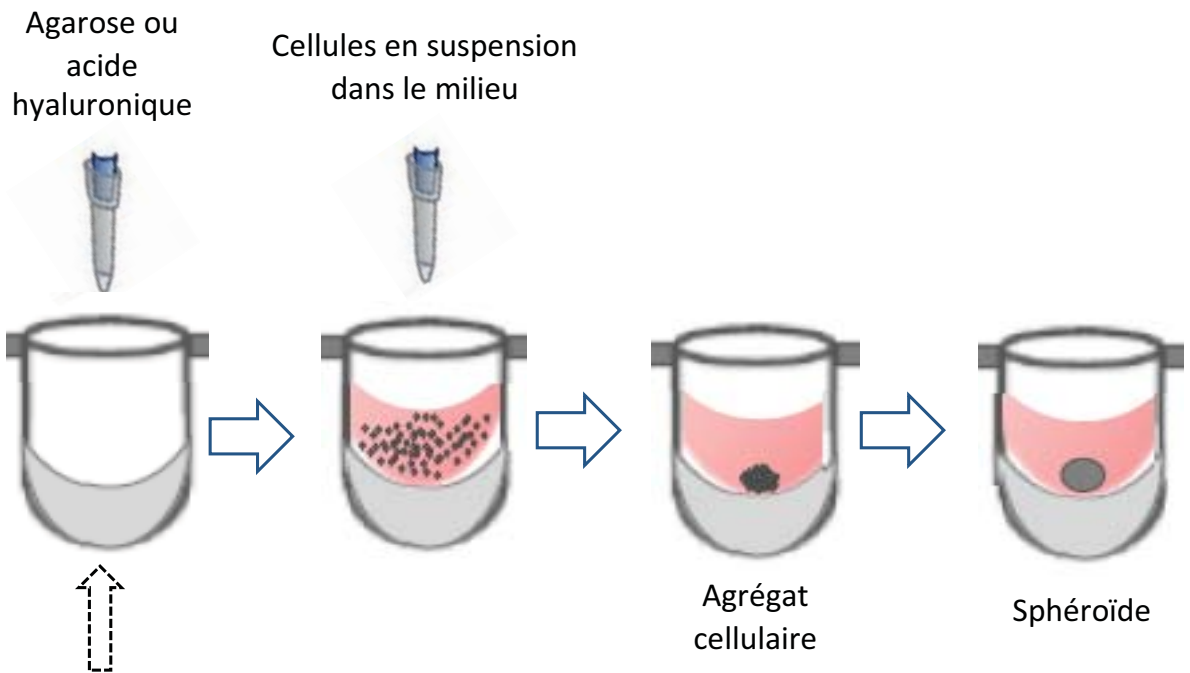
tester le comportement des médicaments avec un bio mimétisme beaucoup plus proche que les techniques conventionnelles de culture en monocouche.

La matrice isolée à partir des cellules **EHS**, est commercialisée sous forme de gel (liquide et soluble à 4°C) avec l'appellation de **Matrigel**® (Corning) (**Kleinman et Martin 2005**). Le **Matrigel**® contient une concentration stable en protéines matricielles (de 10 à 15 mg/mL de protéines). A la température physiologique, celui-ci polymérise afin de reproduire la matrice biologiquement active, semblable à la membrane basale des cellules de mammifères. Des variants du **Matrigel**® dépourvus de facteurs de croissance ou hautement concentrés en collagène IV ont été développés pour des applications spécifiques. Un autre dérivé du **Matrigel**® produit à partir de souris âgées, permet de reproduire le microenvironnement cellulaire dans un contexte de vieillissement (**Kleinman et Martin 2005**). Le **Matrigel**® est recommandé pour la formation, la prolifération et le maintien des cultures cellulaires en 3D produites à partir d'un très grand nombre de types cellulaires (**Nyga, Cheema, et Loizidou 2011; Shin et al. 2013; Kleinman et Martin 2005; Costa et al. 2017**). D'autres solutions concurrentes du **Matrigel** ont été commercialisées en parallèle, telles que le **Geltrex**® (Gibco) ou le **VitroGel**® (Thewellbio®).

❖ Différentes méthodologies de fabrication de cultures cellulaires 3D avec sous sans échafaudage

- Culture 3D avec échafaudage

Les méthodes de formation de sphéroïdes par **échafaudage cellulaire** (ou techniques « *scaffold* ») requièrent l'usage de micro-moules ou micro-réseaux d'hydrogels, de fibres ou de billes au sein desquels les cellules sont scellées. En effet, ces échafaudages créent un réseau plus ou moins dense, propice aux contacts et à la migration cellulaire, recréant les caractéristiques mécaniques de la matrice extracellulaire (**Figure 72**). Lorsque les cellules sont injectées dans de telles structures, elles adhèrent dans un premier temps à la matrice et migrent ensuite jusqu'à l'intérieur du réseau afin de former un micro-tissu. Les différents



OU plaques « **Ultra-Low-Attachment** » (Costar, Corning®)
revêtement hydrophile non toxique, inerte, non dégradabile

Figure 73 : Technique de formation des sphéroïdes *scaffold-free* par « Liquid Overlay » (à partir de Lin, 2008)

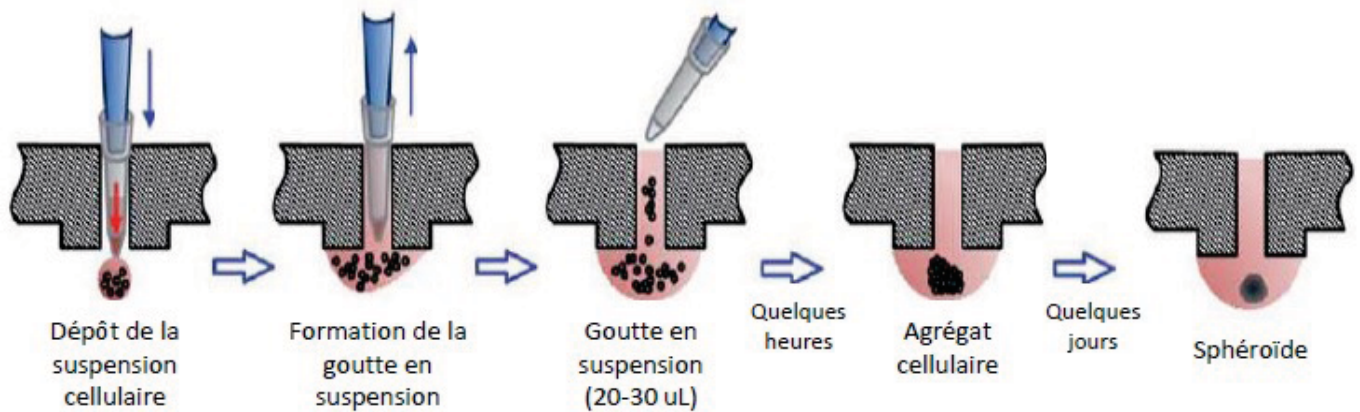


Figure 74 : Technique de formation des sphéroïdes *scaffold-free* par « Hanging Drop » (d'après Tung 2011)

réseaux sont composés de collagène 1, d'acide hyaluronique, de poly-éthylène-glycol (PEG) ou de protéines de soie (**Nyga, Cheema, et Loizidou 2011**). Ces méthodes sont généralement peu reproductibles, de par la difficulté de reproduire un réseau matriciel identique entre les différentes manipulations, influençant la morphologie et topologie des sphéroïdes formés. Par ailleurs, certaines molécules thérapeutiques peuvent s'adsorber sur le réseau matriciel dense, engendrant leur limitation d'accès aux cellules constitutives des sphéroïdes. Finalement, cette méthodologie ne facilite pas l'extraction et l'imagerie des sphéroïdes ainsi formés. En effet, l'échafaudage matriciel dense peut compliquer les observations en microscopie (selon son épaisseur et son opacité), ainsi que l'isolation des sphéroïdes pour des analyses ultérieures.

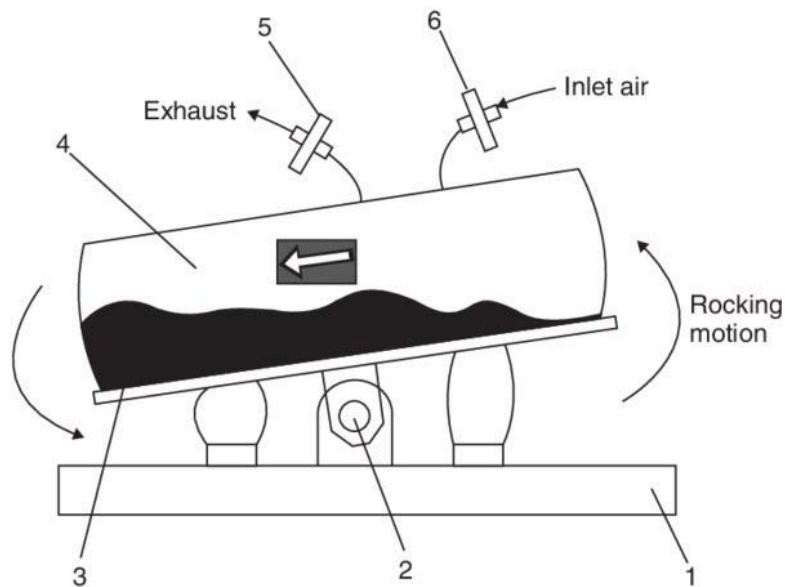
- **Culture 3D sans échafaudage**

Les méthodologies de formation des sphéroïdes sans échafaudages utilisent (1) la gravité (technique *Hanging drop**, technique *liquid overlay**) ou (2) l'agitation constante (technique *Spinner flask* ou en bioréacteurs) (**Charoen et al. 2014; Tung et al. 2011; Costa et al. 2014; Lin, Lin, et Chang 2008; Costa et al. 2017**).

1. Les méthodes d'agrégation cellulaire par **gravité** nécessitent l'utilisation de supports inertes empêchant l'adhésion cellulaire. Parmi ces techniques, les plus connues et développées sont (a) les techniques dites de « **liquid overlay** », utilisant des supports recouverts de films « antiadhésifs » et (b) les techniques de suspension cellulaire au sein de gouttes de culture, telle que la technique « **Hanging Drop** ».

a. La technique **Liquid Overlay** nécessite de recouvrir le fonds des puits ou des boîtes de Pétri avec des protéines limitant l'adhésion des cellules au support, tels que l'agarose, l'acide hyaluronique ou du polyHEMA (**Breslin et O'Driscoll 2016**). Les cellules se déposent alors par gravité dans le fonds des puits sans y adhérer, et s'agrègent naturellement (**Figure 73**).

b. Pour les techniques de culture en suspension, les cellules sontensemencées sur des plaques de culture contenant des puits « sans fonds » (plaques



cellulaire (technologie « Wave »)
 (https://www.researchgate.net/publication/289919455_Cell_culture_bioreactor_s_commercial_production_Encyclopedia_of_Industrial_Biotechnology/figures?lo=1)

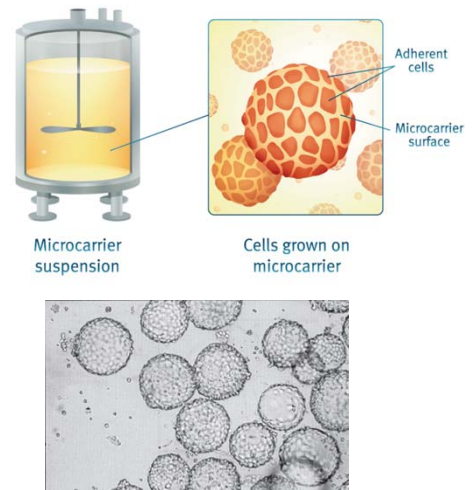
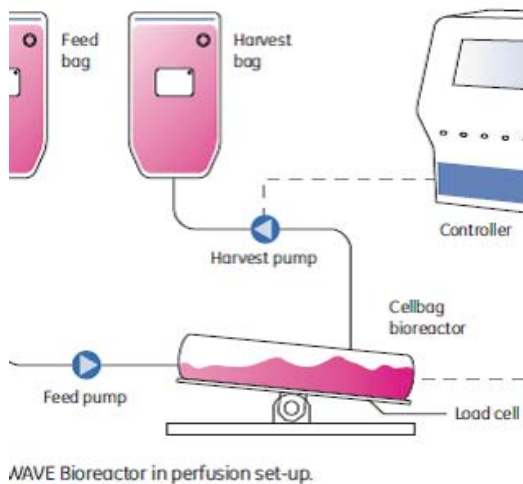


Figure 77 : Technologie de bioréacteur à agitation horizontale et en perfusion constante, permettant d'augmenter la survie et la prolifération cellulaires
 (<http://www.processdevelopmentforum.com/articles/highly-efficient-inoculum-propagation-in-perfusion-culture-using-wave-bioreactor-systems/>)

Figure 78 : culture de cellules adhérentes en bioréacteurs sur micro-carriers (Otto-Wilhelm, 2014)
 Chemotec_ <https://chemometec.com/counting-cells-microcarriers/>)

« **Hanging drop** »). La goutte de milieu de culture contenant les cellules va alors être retenue autour de chaque « pseudo puits » par des forces de rétention. Les cellules tombent ensuite dans le fond de la goutte par gravité et s'agrègent (**Figure 74**).

Pour les deux méthodes, l'agrégation cellulaire induite par la **gravité** et la présence des protéines matricielles créent une forte proximité des cellules favorisant les échanges paracrines et l'initiation des liaisons intercellulaires et cellules/matrice. Ces techniques présentent les avantages d'être peu coûteuses et faciles à mettre en place. Par ailleurs, elles permettent la formation d'un sphéroïde unique par puits, dont la taille (nombre de cellules contrôlé) et la morphologie peuvent être extrêmement maîtrisées. Ces techniques sont utilisées pour un très grand nombre de modèles de cultures cellulaires 3D (**Costa et al. 2014, 2017**) et notamment en Oncologie (**Costa et al. 2017**).

2. Les méthodes de formation de sphéroïdes par **agitation** consistent à maintenir les cellules dans un état de mouvement permanent, limitant leur sédimentation et donc leur adhésion au support de culture. Cette agitation peut être réalisée dans des flasques de culture au sein desquelles de pâles rotatives sont insérées. Ces dernières génèrent un courant permanent dans la culture (*spinner flasks*) (**Figure 75**), ou bien par agitation constante horizontale ou verticale du support de culture placé sur des plateformes mobiles (**Figure 76**). Cependant, ces techniques induisent de fortes forces de cisaillement risquant d'endommager les cellules fragiles. Afin de palier à ces problèmes, de nouvelles méthodologies utilisant des bioréacteurs à perfusion constante ont été développées (**Figure 77**). Ces bioréacteurs permettent notamment d'améliorer l'oxygénation cellulaire et donc de réduire l'impact de ces forces de cisaillement sur la survie et la prolifération cellulaire.

Généralement, afin de former des sphéroïdes à partir de culture de cellules en suspension, des microbilles encore appelées *microcarriers* sont utilisées. Celles-ci permettent l'adhésion des cellules sur ces supports mobiles et facilitent la formation de sphéroïdes (**Gupta et al. 2016; Merten 2015**) (**Figure 78**).

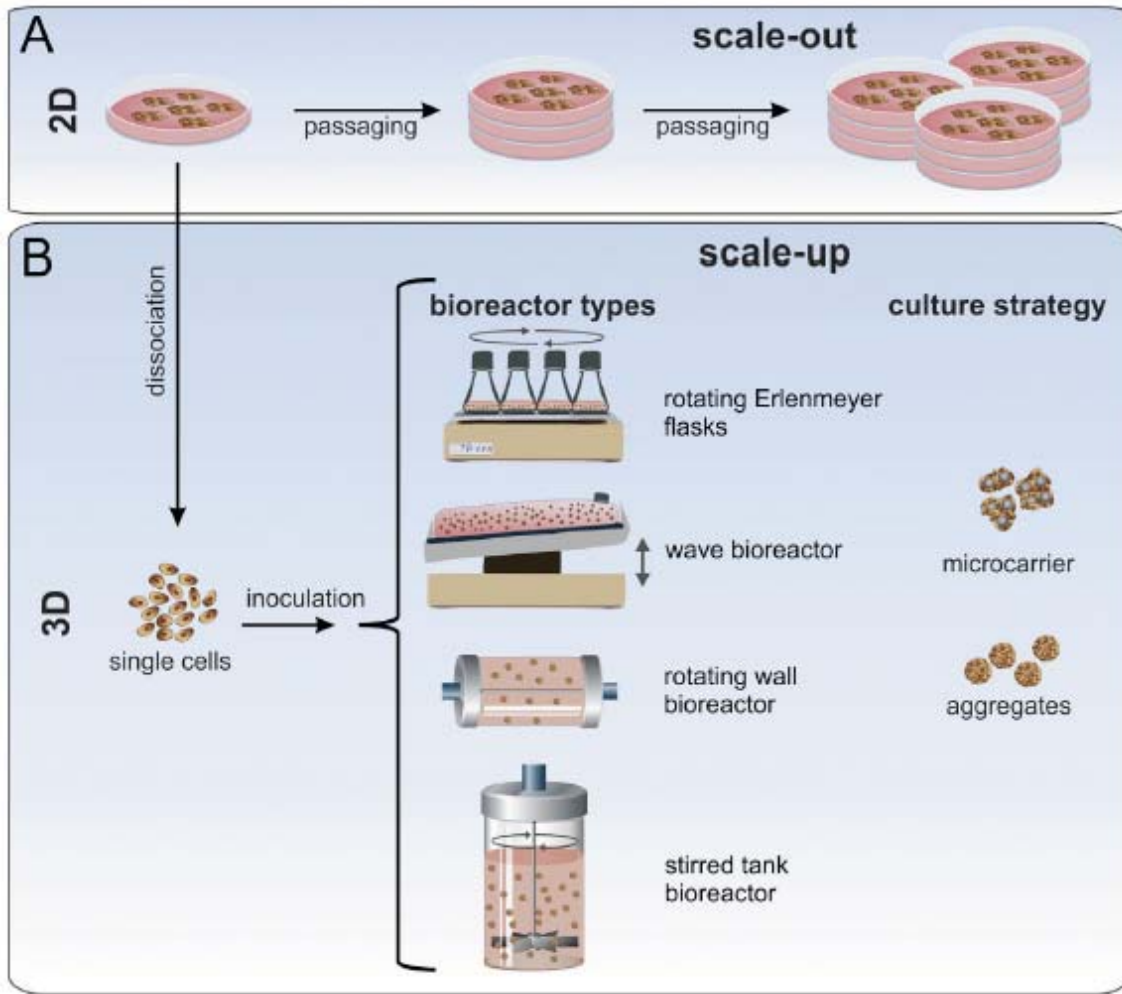


Figure 79 : Changement d'échelle de production cellulaire, de la culture en flasques ou boîtes de pétri à la production à très grande échelle en bioréacteurs (Kropp, 2017)

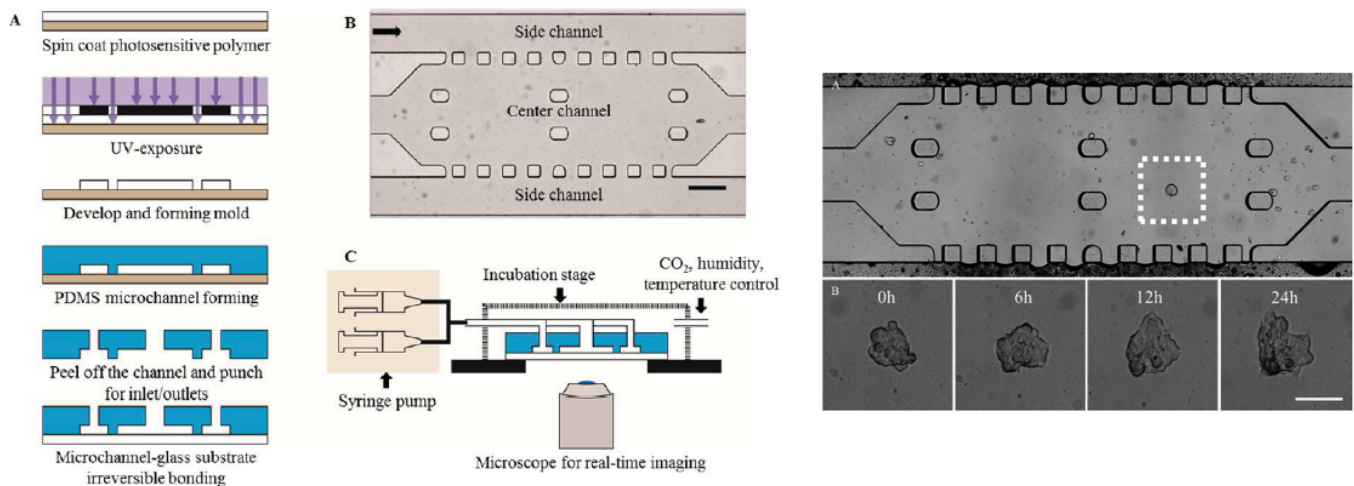


Figure 80 : représentation schématique des techniques de micro-fluidiques adaptées à la culture 3D (Shin, 2013)

Ces technologies utilisant des bioréacteurs ainsi que l'usage des *microcarriers* permettent un très haut rendement de production de sphéroïdes (jusqu'à 100 000 sphéroïdes / mL). (**Massai et al. 2016**), et sont généralement mises en place dans des stratégies de culture à très grande échelle (*scale-up* de production) (**Figure 79**).

D'autres techniques pouvant combiner les deux principales approches ont également été développées afin d'améliorer les modèles de cultures en 3D. Ceux-ci utilisent la **micro-fluidique**, nécessitant l'usage de micro-puces gravées dans des moules ultra denses d'agarose ou de polyméthylsiloxane (PDMS). Ces micro-puces, sur lesquelles sont déposés les sphéroïdes (produits par la technique *scaffold* ou *scaffold-free*), permettent de reproduire les flux d'entrée des nutriments et de sortie des déchets cellulaires et métabolites (**Figure 80**). Bien que très prédictives, ces techniques sont difficilement adaptables pour des cultures à grande échelle et leur mise en place très onéreuse nécessite une expertise longue et complexe.

L'ensemble de ces techniques d'analyse peuvent être utilisées afin de répondre à des questions biologiques majeures, telle que la toxicité/efficacité des thérapies en développement ou en phase d'optimisation.

II) Intégration de la culture 3D dans la modélisation et le screening des agents thérapeutiques en Oncologie

En Oncologie, la culture 3D et plus précisément la technique *Liquid Overlay* (**LOT**) est couramment utilisée afin de caractériser : le nombre de cellules, la viabilité, la morphologie, la prolifération et la différenciation des cellules tumorales, la réponse à des stimuli, la migration et l'invasion, l'angiogenèse, l'évasion du système immunitaire, le métabolisme des médicaments, l'expression génique et la synthèse protéique (**Antoni et al. 2015 ; Lama et al. 2013**). Aussi, de par son plus grand mimétisme biologique et grâce au développement d'outils et de méthodes d'analyses adaptés, celle-ci est de plus en plus intégrée dans les processus de développement des médicaments. En effet, dans les *pipelines* de développement, la culture

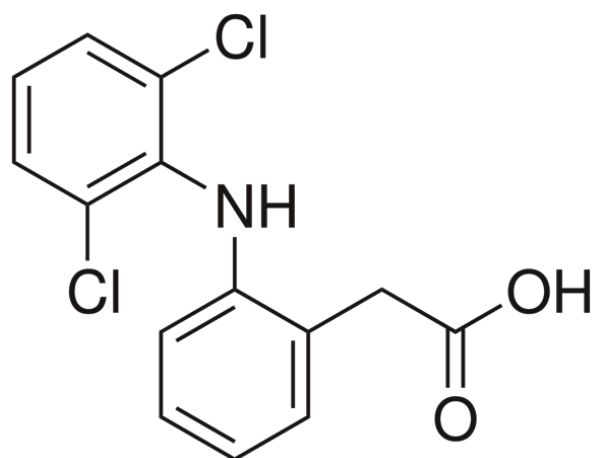


Figure 81 : Structure du Diclofénac

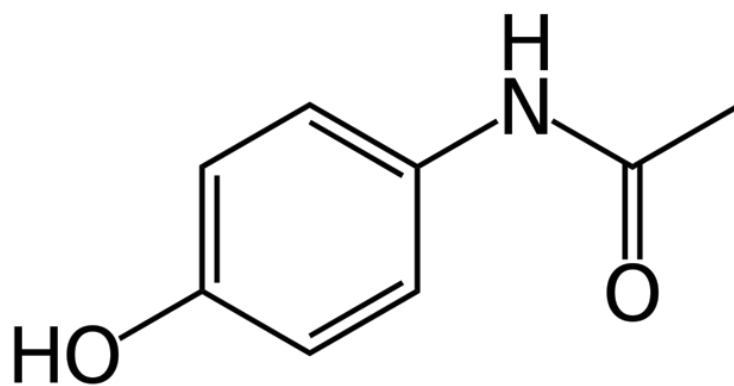


Figure 82 : Structure du Paracétamol

cellulaire 3D se place après les tests cellulaires en 2D, et avant les essais précliniques *in vivo* (expérimentation animale)(**Hirschhaeuser et al. 2010**). Ainsi, grâce à cette technique innovante, un certain nombre de médicaments ont déjà été développés ou optimisés, dans des approches « *bench to bed* » (« de la paillasse au lit du patient ») mais également « *bed to bench* » (« du lit du patient au retour à la paillasse »).

Depuis une trentaine d'années, différentes molécules thérapeutiques ont été étudiées sur des modèles de cultures cellulaires 3D dans des protocoles de développement précliniques, mais également dans des approches d'optimisation des traitements déjà mis sur le marché. Ainsi, grâce aux sphéroïdes formés avec la technique **LOT**, le docétaxel et la doxorubicine ont été étudiés sur des modèles de cancer utérins (**Mikhail, Eetezadi, et Allen 2013**), le cisplatine sur des modèles de cancers du sein, ovariens ou de l'endomètre (**Kobayashi et al. 1993; Chitcholtan, Sykes, et Evans 2012**), le 5-fluorouracile sur des modèles de tumeurs pancréatiques (**Wen et al. 2013**) ou le Tamoxifène sur des modèles de cancers du sein (**Ho et al. 2012**). Par ailleurs, ces modèles de culture 3D sont également utilisés pour caractériser la toxicité secondaire des drogues sur des tissus sains. En effet, la toxicité du diclofénac (**Figure 81**) ou du paracétamol (**Figure 82**) a par exemple été étudiée sur des tissus hépatiques obtenus de prélèvements de patients et cultivés en 3D (**Bell et al. 2016**)(**Gaskell et al. 2016**)(**Costa et al. 2017**). Finalement, un grand nombre d'inhibiteurs de **PARP**, et notamment l'Olaparib et le Rucaparib sont évalués sur des modèles de tumeurs telles que des tumeurs prostatiques (**Rae et Mairs 2017**).

Objectifs des travaux

Les travaux de recherche du groupe « Résistance » de l'équipe 2 de l'UMR INSERM 1240 **IMoST** (Imagerie Moléculaire et Stratégies Théranostiques) sont orientés sur l'optimisation du traitement du cancer du sein TN. Par des approches *in vitro*, ils modélisent l'équilibre de la sensibilité/chimiorésistance (*via* la réponse des protéines MDR) de différentes lignées du cancer du sein TN, en réponse aux thérapies ciblées, notamment les anti-PARPs. Sur cette approche, nos travaux antérieurs se sont portés sur l'étude de l'action/efficacité d'un anti-PARP modèle l'Olaparib, sur la lignée SUM1315 de phénotype TN, en corrélation avec la réponse de deux protéines MDR, la BCRP et P-gp. Ces travaux ont mis en évidence chez la lignée SUM1315 en condition 2D traitée avec Olaparib (50 μ M, faible cytotoxicité), une augmentation de la co-expression de la BCRP et de la P-gp durant 24h de traitement, avec une réponse de type relai. Après 24h, vu la faible toxicité de la dose testée, le taux d'expression des deux protéines MDR retourne à l'état basal. Cependant, l'Olaparib toujours maintenu dans les cellules SUM1315 induirait une nette mortalité cellulaire à long-terme (taux de survie cellulaire : 90% à 24h et 40% à 120h). Ces résultats suggèrent que le mécanisme de résistance des pompes d'efflux MDR peut être contourné par le maintien d'une concentration peu toxique de l'anti-PARP dans les cellules TN mais à long-terme (Dufour et al. 2015). Cependant ces résultats ont été modélisés à partir de cultures cellulaires en monocouche qui ne miment pas la confirmation réelle des tumeurs. Notre groupe de recherche a donc orienté ses travaux sur le développement des modèles plus prédictifs et proche des tumeurs *in vivo*, notamment des cultures cellulaires en trois dimensions.

Dans ce contexte, les travaux de recherche développés au cours de ma thèse ont été orientés sur le développement d'outils diagnostic permettant d'optimiser le traitement des tumeurs mammaires triple-négatives. Ces travaux ont porté sur :

1. Le développement et la caractérisation des cultures cellulaires en 3D à partir de deux lignées du cancer du sein Triple Négatif, SUM1315 et MDA-MB-231. Ces travaux ont fait l'objet d'une **publication en premier auteur parue dans la revue *Oncotarget* (IF 5,2)**. Par la suite, afin d'augmenter la reproductibilité des cultures cellulaires 3D nous avons recherché à développer des conditions normalisées de culture, en limitant l'usage d'éléments d'origine animale. En effet, dans le cadre d'une collaboration de

recherche public/privé, nous avons adapté un milieu de culture innovant synthétique sans sérum de veau ajouté intitulé **OptiPASS** (société BIOPASS), à ces deux modèles de cultures 3D. Ces travaux sont valorisés par une ***publication en premier auteur soumise dans la revue Experimental Cell Research.***

2. L'optimisation du traitement des tumeurs mammaires TN par la modélisation de l'efficacité d'un traitement combinant l'Olaparib (anti-PARP1) et la radiothérapie fractionnée, en conditions de culture 2D et 3D. Ces travaux ont été effectués en collaboration avec le service de Radiothérapie et le département de Physique Médicale du Centre Jean Perrin et seront valorisés par une ***publication en premier auteur en cours de soumission.***
3. Le développement d'un outils diagnostic dérivé de l'Olaparib et couplé à un fluorochrome (Ola-FL) permettant d'étudier/analyser le degré de pénétration/d'efficacité/de cytotoxicité des anti-PARPs sur des modèles de cultures cellulaires 3D. Ces travaux font l'objet d'une ***publication en premier auteur.*** Parallèlement, une méthode de quantification de l'Olaparib intracellulaire a été ***publiée en troisième auteur dans la revue Journal of Pharmaceutical and Biomedical Analysis (IF 2,8).***
4. La caractérisation de la chimiorésistance induite par l'Olaparib sur les modèles 2D et 3D par l'analyse de l'expression des protéines de *MultiDrug Resistance* MRP7 et P-gp. Ces travaux font l'objet d'une ***publication en premier auteur.***
5. Enfin, dans le cadre d'une collaboration de recherche avec l'UMR 6533 CNRS/IN2P3, LPC+, Equipe Physico Chimie des Surfaces Nanostructurées, l'étude de la toxicité de nanoparticules d'or PEGYlées sur la lignée cellulaire TN HCC- 1937 a été réalisée. Ces travaux ont fait l'objet d'une ***publication en deuxième auteur parue dans la revue Journal of Biomaterials and Nanobiotechnology (IF 1,4).***

Résultats expérimentaux

A) Développement des cultures cellulaires en trois dimensions à partir des lignées cellulaires MDA-MB-231 et SUM1315 issues de cancers du sein Triple-Négatifs

I) Introduction

Le processus de développement de composés à usage thérapeutique se décline en phases de Recherche préclinique suivies de phases d'essais cliniques, chez l'Homme (**Commissioner s. d.**). Ce processus est extrêmement long, exigeant et coûteux. De plus, seulement un composé sur 5000 testés obtiendrait une autorisation pour une mise sur le marché pour être utilisé en clinique (**Commissioner s. d.**). Néanmoins, ces différentes étapes sont indispensables afin de choisir un médicament, de tester son efficacité et de s'assurer de la sécurité de ce composé en vue de son usage chez l'Homme.

Les tests précliniques sont composés d'essais primaires et secondaires. Les essais primaires englobent des tests de « screenings » à haut débit permettant d'isoler les composés présentant un intérêt biologique potentiel pour une pathologie. Ces étapes de « screening » peuvent être réalisées *in silico* et/ou *in vitro*, à l'aide des tests enzymatiques et/ou à partir de cultures cellulaires (**Hirschhaeuser et al. 2010**). Ensuite, des tests secondaires plus précis visant à déterminer la toxicité, le métabolisme et la biodisponibilité des molécules d'intérêt seront réalisés à l'aide des cultures cellulaires et des modèles animaux (**Hirschhaeuser et al. 2010**). Cependant, plusieurs questions éthiques et économiques se posent quant à l'utilisation des différents modèles. En effet, depuis un dizaine d'années, des mesures restrictives ont été mises en place afin de réduire, raffiner voire remplacer l'expérimentation animale dans les processus de développement des médicaments (« **La règle des 3 R : réduire, raffiner, remplacer** » **s. d.; Balls 2009**). Parallèlement, l'usage des cultures cellulaires *in vitro* en monocouche fait l'objet d'une importante remise en question, notamment en termes de manque de biomimétisme. En effet, un grand nombre d'études a démontré la faible efficacité de prédiction de l'action des molécules testées sur des cultures cellulaires 2D en comparaison des modèles animaux et de leur action chez l'Homme (**Breslin et O'Driscoll 2016; Duval et al.**

2017; Imamura et al. 2015). Ainsi, dans l'espoir d'augmenter la prédiction des tests *in vitro* tout en limitant les coûts de développement des médicaments, de nouveaux modèles d'étude précliniques ont été développés, et notamment la culture cellulaire en trois dimensions (culture 3D).

Dans ce contexte, le premier objectif de ces travaux de thèse portait sur le développement des cultures cellulaires en trois dimensions à partir de plusieurs lignées de tumeurs TN, et notamment des deux lignées cellulaires **MDA-MB-231** et **SUM1315**. Pour ces travaux, la technique de culture en 3D par « liquid overlay », très reproductible, a été utilisée (**LOT**). Elle consiste à cultiver les cellules dans des puits à fond conique recouverts d'un revêtement spécifique (polymères) empêchant l'adhésion des cellules (Technologie « Ultra-Low-Attachment »). Suite à l'ensemencement, l'agrégation des cellules est effectuée pendant un temps d'incubation défini dans les puits. Ensuite, les protéines matricielles (Geltrex^{TM*}) sont rajoutées au milieu de culture. Afin de déterminer les conditions optimales de culture, différents paramètres ont été testés tels que (i) des concentrations cellulaires d'ensemencement croissantes, (ii) des concentrations en protéines matricielles croissantes et (iii) différentes conditions d'addition des protéines matricielles. Par la suite, sur les deux modèles de sphéroïdes MDA-MB-231 et SUM1315 ainsi optimisés et standardisés, des études (i) de suivi de la prolifération, (ii) de l'évolution de l'activité métabolique, (iii) et des profils de viabilité/mortalité des sphéroïdes ont été effectuées durant 14 jours. Parallèlement, la topologie et l'ultrastructure des sphéroïdes ont été analysées par microscopie électronique à balayage (topologie générale tridimensionnelle) et à transmission (recherche d'organites cellulaires intacts dans toute la profondeur du sphéroïde et de jonctions matrice/cellules et cellules/cellules). Après le développement et la caractérisation de ces modèles 3D optimisés, leur sensibilité vis à vis de différents chimiothérapeutiques utilisés pour le traitement des tumeurs TN, l'épirubine, du docétaxel et du cisplatine, a également été étudiée. Celle-ci a permis la validation de tests de « screening » développés pour ces modèles de culture cellulaire. **L'ensemble de ces travaux a fait l'objet d'une publication en premier auteur acceptée en Juillet 2017 dans la revue spécialisée Oncotarget (IF 5,255).**

II) (*Article paru*) Development and cytotoxic response of two proliferative MDA-MB-231 and non-proliferative SUM1315 three-dimensional cell culture models of triple-negative basal-like breast cancer cell lines

Development and cytotoxic response of two proliferative MDA-MB-231 and non-proliferative SUM1315 three-dimensional cell culture models of triple-negative basal-like breast cancer cell lines

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ABSTRACT

Triple-Negative Basal-Like tumors, representing 15 to 20% of breast cancers, are very aggressive and with poor prognosis. Targeted therapies have been developed extensively in preclinical and clinical studies to open the way for new treatment strategies. The present study has focused on developing 3D cell cultures from SUM1315 and MDA-MB-231, two triple-negative basal-like (TNBL) breast cancer cell lines, using the liquid overlay technique. Extracellular matrix concentration, cell density, proliferation, cell viability, topology and ultrastructure parameters were determined. The results showed that for both cell lines, the best conditioning regimen for compact and homogeneous spheroid formation was to use 1000 cells per well and 2% Geltrex®. This conditioning regimen highlighted two 3D cell models: non-proliferative SUM1315 spheroids and proliferative MDA-MB-231 spheroids. In both cell lines, the comparison of 2D vs 3D cell culture viability in the presence of increasing concentrations of chemotherapeutic agents *i.e.* cisplatin, docetaxel and epirubicin, showed that spheroids were clearly less sensitive than monolayer cell cultures. Moreover, a proliferative or non-proliferative 3D cell line property would enable determination of cytotoxic and/or cytostatic drug activity. 3D cell culture could be an excellent tool in addition to the arsenal of techniques currently used in preclinical studies.

INTRODUCTION

Triple-negative basal-like (TNBL) tumors comprise 70% of the basal-like tumor subtype and 15% of all breast cancers. They are characterized by the non-expression of estrogen receptors (ER) and progesterone receptors (PR), and the absence of HER2 over-expression or *ERBB-2* amplification [1–4]. TNBL tumors mainly affect young women and are frequently associated with hereditary predispositions (*BRCA1/2* germline mutations). This subtype has a very poor prognosis. TNBL tumours have a high proliferative capacity and may respond well to neoadjuvant chemotherapy or develop a resistance phenotype associated with metastases.

Conventional chemotherapy is based on different protocols such as FEC (5-fluorouracil, epirubicin, cyclophosphamide), FAC (5-fluorouracil, adriamycin, cyclophosphamide) or platinum salts (cisplatin), generally associated with side effects [5]. Otherwise, these tumors are not sensitive to classical breast targeted therapies since they do not express the relevant receptors (*i.e.* ER, PR and HER2). Thus, different groups have aimed to develop alternative targeted therapies. Targeting Epidermal Growth Factor Receptor 1 pathway with anti-EGFR Monoclonal antibodies (MoAb) or Tyrosine Kinase Inhibitors (TKI); or inhibiting the enzyme Poly-ADP-Ribose-Polymerase1 (reparation of single-strand breaks)(PARPi) have shown promising activities in this preclinical and clinical setting [5–8].

Monolayer *in vitro* cell culture studies represent a gold standard high throughput screening for toxicity of chemotherapeutics. However, this type of culture does not reproduce the three-dimensional (3D) structural properties of tumors. In fact, these tumors are biochemically and structurally characterized by (i) the generation of hypoxic regions, (ii) intercellular interactions, (iii) nutrient and growth factor exchanges, and (iv) the production of extracellular matrix that is essential to tumor stability and regulation of cellular functions [9–11].

Therefore, over recent decades, 3D cell culture, mimicking the 3D organization of *in vivo*-like tumors, has regained interest. This innovative technique consists in forming aggregated and compact cell clusters called spheroids. In this conformation, cells gain different characteristics depending on their position [9]. Variable gradients develop for oxygen, nutrients, lactate accumulation and pH [9]. The easily accessible peripheral cells are metabolically active and able to proliferate. Conversely, cells in the centre have unfavourable conditions and ultimately become quiescent and form a necrotic core [12, 13]. Taken together these metabolic and biochemical heterogeneous features reproduce the inconstancy of tumors with respect to their response to treatment and predict more accurately the features of *in vivo* tumors [14]. Several 3D culture methods are available based on (i) the induction of mechanical forces

i.e. centrifugation pellet culture, spinner flask culture and rotary cell culture systems, (ii) micromolding in hydrogels and (iii) gravity *i.e.* hanging drop culture and liquid overlay culture [13, 15, 16]. Amongst these methods, the liquid overlay technique uses cells capacity to aggregate by gravity on non-adherent surfaces and form spheroids within a short period of time.

The 3D cell culture model is being increasingly integrated into the drug development process as it appears to be a more accurate model when studying the impact of chemotherapeutics [17]. In fact, in terms of predicting potential drug efficacy, it is currently better than 2D cell culture [9].

This work focuses on the development of a 3D cell culture from two TNBL breast cancer cell lines, SUM1315 and MDA-MB-231, using the “liquid overlay” technique. For this, spheroid extracellular matrix concentration, cell density, metabolic activity, cell viability, proliferation, topology and ultrastructure parameters were firstly determined for both cell lines. Then, in order to better characterize 3D cell culture response, cell drugs sensibility of these spheroids was analysed in comparison to 2D cell culture.

RESULTS

3D cell culture development from TNBL cell lines

3D cell culture development was carried out using two TNBL cell lines, SUM1315 and MDA-MB-231. For each cell line, four parameters were determined: (i) extracellular matrix protein concentration (ii) cell spheroid concentration, (iii) spheroid cell metabolic activity and (iv) spheroid cell viability/mortality.

SUM1315 cell line

Extracellular matrix protein concentration

For these experiments, Geltrex® was used as extracellular matrix proteins. SUM1315 cells were seeded at 5000 cells per well in “Ultra-Low-Attachment” microplates (Corning®) at “Day 0”. Then, several conditioning regimens of Geltrex® were tested, *i.e.* just after cell seeding or 24h after cell seeding, and several Geltrex® concentrations (0.25 to 6%) were tested (Table 1, Figure 1B, 1C) and compared with a control cell culture without Geltrex® (Figure 1A).

The results showed that when Geltrex® was added just after cell seeding (at Day 0), loose cell aggregates were detected in the presence of 0.25 and 0.5% of Geltrex® concentrations (Table 1, Figure 1A). In the presence of 1 to 6% Geltrex®, only scattered spheroids were detected (Table 1, Figure 1B). When Geltrex® was added 24h after cell seeding (at Day 1), loose aggregates and scattered spheroids were observed in the presence of 0.25 to 0.5%

Table 1: Extracellular matrix concentration determination for spheroid formation with the SUM1315 cell line

Percentage of Geltrex in the culture medium	Condition of Geltrex® adding	
	After cell seeding (Day 0)	24h after cell seeding once aggregate formation (Day 1)
0 (control)	Unique loose aggregate	Unique loose aggregate
0.25 - 0.5	Unique loose aggregate	Unique loose aggregate
1 - 2	Scattered spheroids	Unique compact spheroid
3 - 4 - 5 - 6	Scattered spheroids	Scattered spheroids

Several Geltrex® concentrations from 0.25 to 6% were used. Two conditioning regimens of Geltrex® were tested: addition of Geltrex® (i) in the microplates just after cell seeding (corresponding to Day 0) and (ii) 24h after cell seeding and aggregate formation (corresponding to Day 1).

and 3 to 6% Geltrex® concentrations, respectively (Table 1, Figure 1A, 1B). In contrast, single compact spheroids were detected with 1 to 2% Geltrex® added 24h after cell seeding (Table 1, Figure 1C). Therefore 2% Geltrex® added at Day 1 was the conditioning regimen chosen for 3D culture development of the SUM1315 cell line.

Cell spheroid concentration determination

SUM1315 cell concentrations ranging from 50 to 10000 cells per well were cultured in the presence of 2% Geltrex® added after cell seeding at Day 1 (Figure 2A). Spheroid diameters were monitored every day from Day 0 (control) to Day 14 with light microscopy, using ToupView® software. Results showed that for 50, 2000 and 10000 cells per well, spheroid diameters were $299 \pm 54 \mu\text{m}$, $1198 \pm 45 \mu\text{m}$ and $2149 \pm 50 \mu\text{m}$ at Day 0 and decreased to $89 \pm 16 \mu\text{m}$, $320 \pm 21 \mu\text{m}$ and $570 \pm 21 \mu\text{m}$ at Day 1, respectively. SUM1315 spheroid diameters and shapes remained constant until Day 14 for concentrations of 200 to 2000 cells per well ($313 \pm 14 \mu\text{m}$ for 2000 cells per well), and decreased for concentrations of 5000 and 10000 cells per well ($401 \pm 31 \mu\text{m}$ for 10000 cells, $p < 0.00001$) (Figure 2A, 2B).

Metabolic activity study in 3D cell culture

Metabolic activity was measured by the colorimetric resazurin test involving the reduction of resazurin into resorufin. For the SUM1315 cell line, this parameter was analysed at Day 1, Day 8 and Day 14 in spheroids of 50 to 10000 cells per well. Optic density (OD) of resorufin increased significantly for concentrations of 50, 200, 1000 and 2000 cells per well from Day 1 (with 0.006 ± 0.002 , 0.007 ± 0.003 , 0.051 ± 0.008 and 0.120 ± 0.044 , respectively) to Day 8 (with 0.084 ± 0.021 ($p < 0.00001$), 0.076 ± 0.022 ($p < 0.001$), 0.179 ± 0.015 ($p < 0.00001$) and 0.233 ± 0.043 ($p < 0.01$, respectively)) (Figure 2B–2C). For cell concentrations of 5000 and 10000 per well, the OD of resorufin remained stable after 8 days of culture, with OD of 0.255 ± 0.025 and 0.306 ± 0.027 at Day 1 compared to 0.275 ± 0.024 ($p = 0.23$) and 0.324 ± 0.028 ($p = 0.73$) at Day 8, respectively.

After Day 8, the metabolic activity of SUM1315 spheroids dropped dramatically for all cell concentrations to Day 14 (OD of 0.047 ± 0.010 for 5000 cells per well) (Figure 2–2C).

Spheroid cell viability/mortality monitoring

SUM1315 spheroid cell viability was analyzed during the experiment (14 days) by fluorescence microscopy using Live/Dead® kit (Molecular Probes). Cells forming spheroids remained viable (green markings) with all studied concentrations (50 to 10000 cells per well) for the 14 days of culture (Figure 2D), with no change in green fluorescent probe intensity between Day 8 and Day 14.

All these results showed that whatever the tested cell concentrations in the presence of 2% Geltrex® added after aggregate formation, compact and homogenous spheroids were formed. Moreover, spheroid diameters and viability remained stable for 14 days with all cell concentrations. Nevertheless, metabolic activity in spheroids decreased dramatically for all cell concentrations after 8 days of culture.

MDA-MB-231 cell line

Extracellular matrix protein concentration

For 3D cell culture development with the MDA-MB-231 cell line, the same Geltrex® concentrations and conditioning regimens were used as above. Single compact spheroids were detected with 2% Geltrex® added 24h after cell seeding (*data not shown*).

Cell spheroid concentration determination

For this cell line, as previously, spheroid diameters obtained with MDA-MB-231 cell concentrations from 50 to 10000 cells per well were measured every day from Day 0 to Day 14 with light microscopy using ToupView® Software (Figure 3A, 3B). At Day 0 for 50, 2000 and 10000 cells per well, diameters were $254 \pm 50 \mu\text{m}$, $1203 \pm 100 \mu\text{m}$ and $2246 \pm 84 \mu\text{m}$ respectively. In contrast, spheroid diameters decreased significantly

SUM 1315

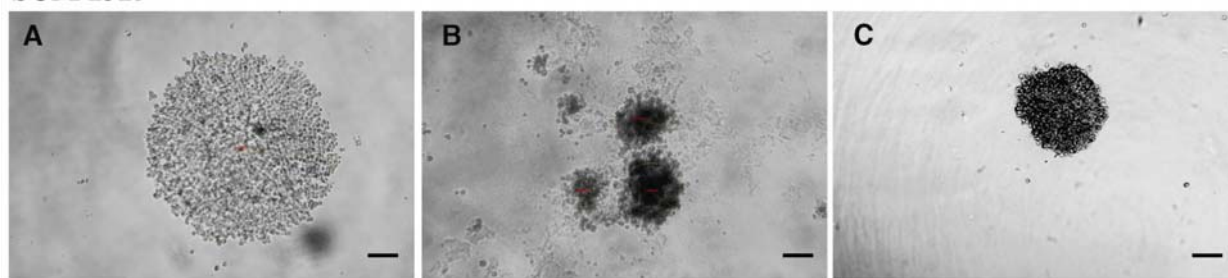


Figure 1: Profile of spheroid formation with the SUM1315 cell line in light microscopy. Aggregates and spheroids were observed by light microscopy at Day 1, using ToupView® software. Spheroid formation was considered when cells were compacted in a unique and opaque to light aggregate. Magnification = 100X, scale bar = 200µm. (A) SUM1315 loose cell aggregates, (B) scattered spheroids, (C) single compact spheroid.

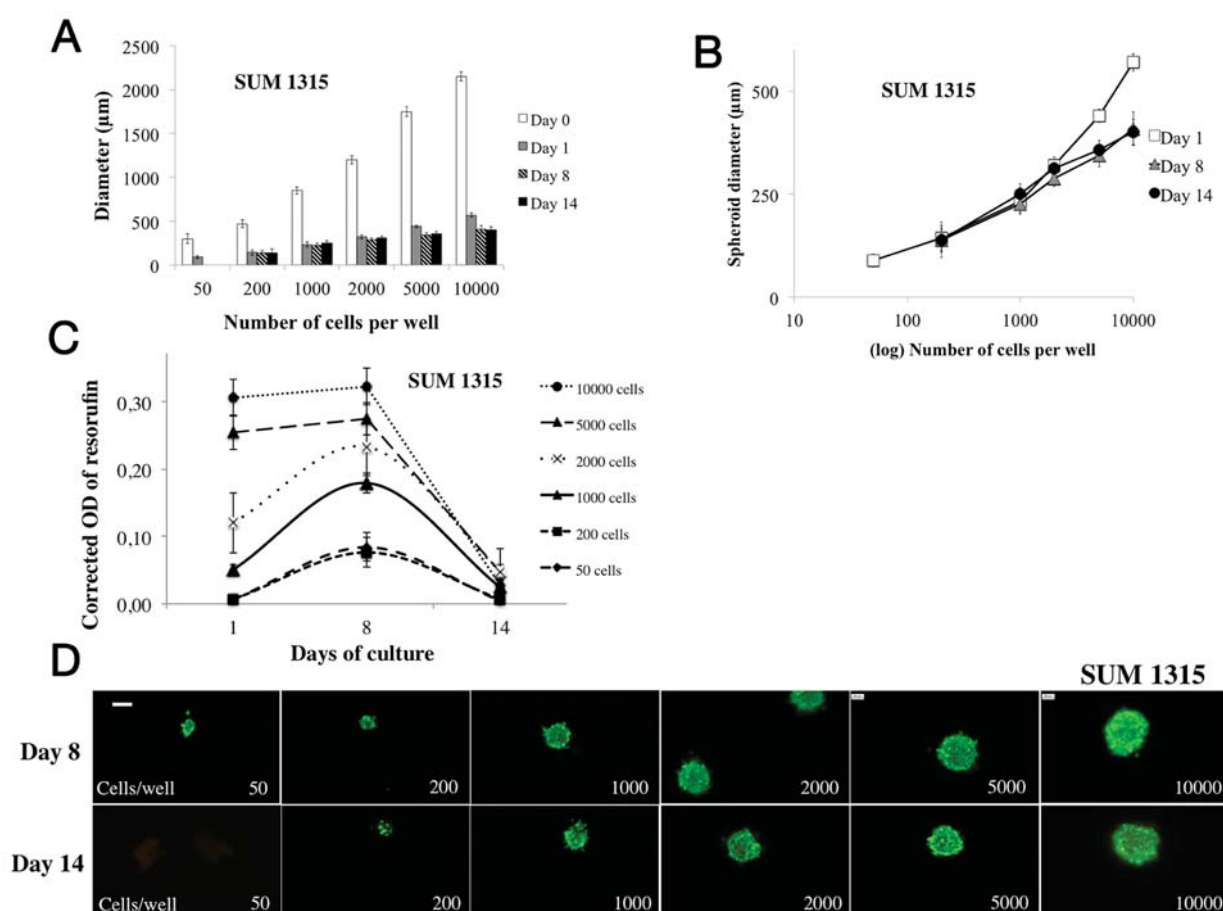


Figure 2: Parameter determination for SUM1315 cell line spheroid formation. For SUM1315 cell line spheroid formation, 50 to 10000 cells were seeded in 96-wells microplates (“ULA” for Ultra Low Attachment, Corning®) at Day 0 and 2% Geltrex® was added to the wells at Day 1. All experiments were carried out at Day 1, Day 8 and Day 14. (A) *Cell concentration determination for spheroid formation*: the diameter of each spheroid was measured (µm) by light microscopy with ToupView® software. Day 0 measurements correspond to aggregate diameters. (B) *Spheroid diameter evolution over time*: spheroid diameter (µm) was measured with ToupView software® for all tested cell concentrations (log10 scale). (C) *Cell metabolic activity in spheroids via resazurin test*: the resazurin is reduced into resorufin by metabolically active cells. Corrected OD of resorufin (λ570- λ620 nm) was measured after 15h incubation with 60 µM resazurin in PBS. (D) *Cell viability/mortality in spheroids with LiveDead® imaging*: Green marking corresponds to calcein-AM penetration (viable cells). Red markings correspond to ethidium homodimer-1 cell penetration (dead cells). Scale bar = 200 µm. Graphs represent at least triplicate biological repeats and are displayed as mean ± SEM and *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 and *****p<0.00001.

after adding Geltrex®. At Day 1, diameters were $134\pm 23\ \mu\text{m}$ ($p<0.00001$), $612\pm 59\ \mu\text{m}$ ($p<0.00001$) and $826\pm 90\ \mu\text{m}$ ($p<0.00001$) for 50, 2000 and 10000 cells per well, respectively. Furthermore, the monitoring of MDA-MB-231 spheroids showed a significant increase in diameters at Day 8 for concentrations of 50 to 2000 cells per well with $317\pm 65\ \mu\text{m}$ ($p<0.00001$) and $843\pm 69\ \mu\text{m}$ ($p<0.00001$), respectively. Moreover, for concentrations of 5000 and 10000 cells per well, no diameter increase was detected under these experimental conditions ($814\pm 67\ \mu\text{m}$ for 10000 cells per well at Day 8, $p=0.68$ and $848\pm 76\ \mu\text{m}$ at Day 14, $p=0.27$) (Figure 3A, 3B). After 14 days, spheroid diameters continued increasing significantly for cell concentrations of 50 to 1000 cells per well ($608\pm 148\ \mu\text{m}$ for 50 cells per well, $p<0.00001$).

Metabolic activity study in 3D cell culture

Metabolic activity assessment was carried out for 14 days, in MDA-MB-231 spheroids seeded with 50 to 10000 cells per well (Figure 3C). The OD of resorufin significantly increased over time with all cell concentrations after 8 days of culture. At Day 1, OD was 0.010 ± 0.003 , 0.062 ± 0.005 and 0.165 ± 0.023 and increased to 0.054 ± 0.006 ($p<0.00001$), 0.115 ± 0.010 ($p<0.00001$) and 0.244 ± 0.035 ($p<0.001$) at Day 8 for 200, 1000 and 10000 cells per well respectively.

The metabolic activity of spheroids continued increasing for spheroids of 50 to 200 cells per well (0.167 ± 0.007 for 200 cells, $p<0.00001$), remained stable for spheroids of 1000 cells per well (0.128 ± 0.014 , $p=0.11$), and decreased significantly for spheroids of 2000, 5000 and 10000 cells per well (0.074 ± 0.037 for 10000 cells, $p<0.00001$ compared to Day 8).

Spheroid cell viability/mortality monitoring

MDA-MB-231 spheroid cell viability was monitored during the experiment (14 days) by fluorescence microscopy using Live/Dead® kit (Molecular Probes) (Figure 3D). Cells forming spheroids remained viable (green markings) with all studied concentrations (50 to 10000 cells per well) for the 8 days of culture. In contrast, at day 14, green markings were less intense (in same conditions of exposure and gamma) with spheroids formed with 2000 to 5000 cells per well, and even absent with 10000 cells per well.

Overall results obtained with the MDA-MB-231 cell line also showed that whatever the tested cell concentrations in the presence of 2% Geltrex® added after aggregate formation, compact and homogenous spheroids were formed. Spheroid diameters increased clearly for concentrations of 50 to 2000 cells per well for 14 days, suggesting a proliferative property of the MDA-MB-231 cell line under these experimental conditions. Moreover, metabolic activity in spheroids increased clearly during 8 days of culture presenting stable viability for all cell concentrations. Nevertheless, for cell concentrations

superior to 2000 per well, metabolic activity and viability (Live/Dead® kit) dropped after 14 days.

For further experiments, with both SUM1315 and MDA-MB-231 spheroid cell cultures, the conditioning regimen of 1000 cells per well and 2% Geltrex® were chosen.

Topological and ultrastructural 3D cell culture characterization

3D cell culture architecture of SUM1315 and MDA-MB-231 spheroids with Scanning Electron Microscopy

Scanning Electron Microscopy (SEM) was used to assess the 3D structure of cells forming the spheroids. Both SUM1315 (Figure 4A, 4B, 4C) and MDA-MB-231 (Figure 4D, 4E, 4F) compact and homogenous spheroids exhibited rounded shapes of about $300\ \mu\text{m}$ with granular surfaces covered with stacked cells. These cells in clusters were $10\ \mu\text{m}$ in diameter and most of them presented rounded morphology. They are all closely juxtaposed with extracellular matrix (ECM) filling the empty spaces.

Ultrastructure of SUM1315 and MDA-MB-231 spheroids with Transmission Electron Microscopy

The organization and ultrastructure of cells forming the spheroids were analyzed using Transmission Electron Microscopy (TEM) of SUM1315 (Figure 5A, 5B, 5C, 5D) and MDA-MB-231 (Figure 5E, 5F, 5G, 5H) spheroids. SUM1315 (Figure 5A) and MDA-MB-231 spheroids (Figure 5E) displayed adjoined cells with intact plasma and nuclear membranes. Cells established contact by two types of cell junctions: tight junctions (Figure 5C, 5G) and anchoring junctions (*zonula adherens*, Figure 5D) as well as desmosomes (Figure 5B, 5H). Ultrastructural analysis at higher magnifications ($M=15000 - 20000\ \text{X}$) revealed the presence of conventional organelles such as mitochondria, rough endoplasmic reticulum and lysosomes (Figure 5B, 5F).

2D and 3D TNBL cell culture metabolic activity comparison

The comparison of metabolic activity between 2D and 3D cell cultures (as described above) was analyzed with the resazurin test after 5 days of culture of SUM1315 (Figure 6A) and MDA-MB-231 (Figure 6B) cell lines seeded at 1000 cells/well. SUM1315 cell metabolic activity in 2D cell culture was $0.098\pm 0.005\ \text{AU}$ and significantly higher than 3D cell culture with $0.053\pm 0.008\ \text{AU}$ ($p<0.00001$) (Figure 6A). Similarly, for the MDA-MB-231 cell line, it was $0.179\pm 0.025\ \text{AU}$ in 2D and significantly higher than 3D cell culture with $0.077\pm 0.013\ \text{AU}$ ($p<0.00001$) (Figure 6B). These results showed that metabolic activity of cells cultured in 3D was lower than cells cultured in monolayer under these experimental conditions.

Analysis of 2D and 3D TNBL cell culture sensitivity to drugs

Sensitivity of SUM1315 and MDA-MB-231 cell lines in 2D and 3D cell cultures (as described above) was studied in the presence of three conventional chemotherapeutic drugs *i.e.* cisplatin, docetaxel and epirubicin. A quantitative resazurin test was used after 5 days of treatment to determine cell viability (Figures 7, 8, 9). The percentage of cell viability was calculated by the ratio of the quantity of resorufin formed by treated cells in comparison with untreated cells. For 3D cell culture, spheroid diameter measurements and qualitative viability/mortality analysis using the Live/Dead® kit were also

performed at Day 5. In parallel, controls and solvent controls with DMSO 0.1% were also performed under the same culture conditions.

Sensitivity to cisplatin

For the SUM1315 cell line, analysis of the ratio of the quantity of resorufin formed in DMSO 0.1% treated cells in comparison with untreated control cells showed no impact of DMSO on cell viability, with $99 \pm 11\%$ for 2D cell culture ($p=0.69$) and $101 \pm 24\%$ for 3D cell culture ($p=0.61$). With cisplatin, the analysis of cell viability in 3D and 2D cell cultures at day 5 was of $80 \pm 14\%$ vs $101 \pm 8\%$ with $0.01 \mu\text{M}$ ($p<0.00001$), $72 \pm 9\%$ vs $104 \pm 5\%$ with $0.1 \mu\text{M}$ ($p<0.00001$), $74 \pm 9\%$ vs $103 \pm 4\%$ with

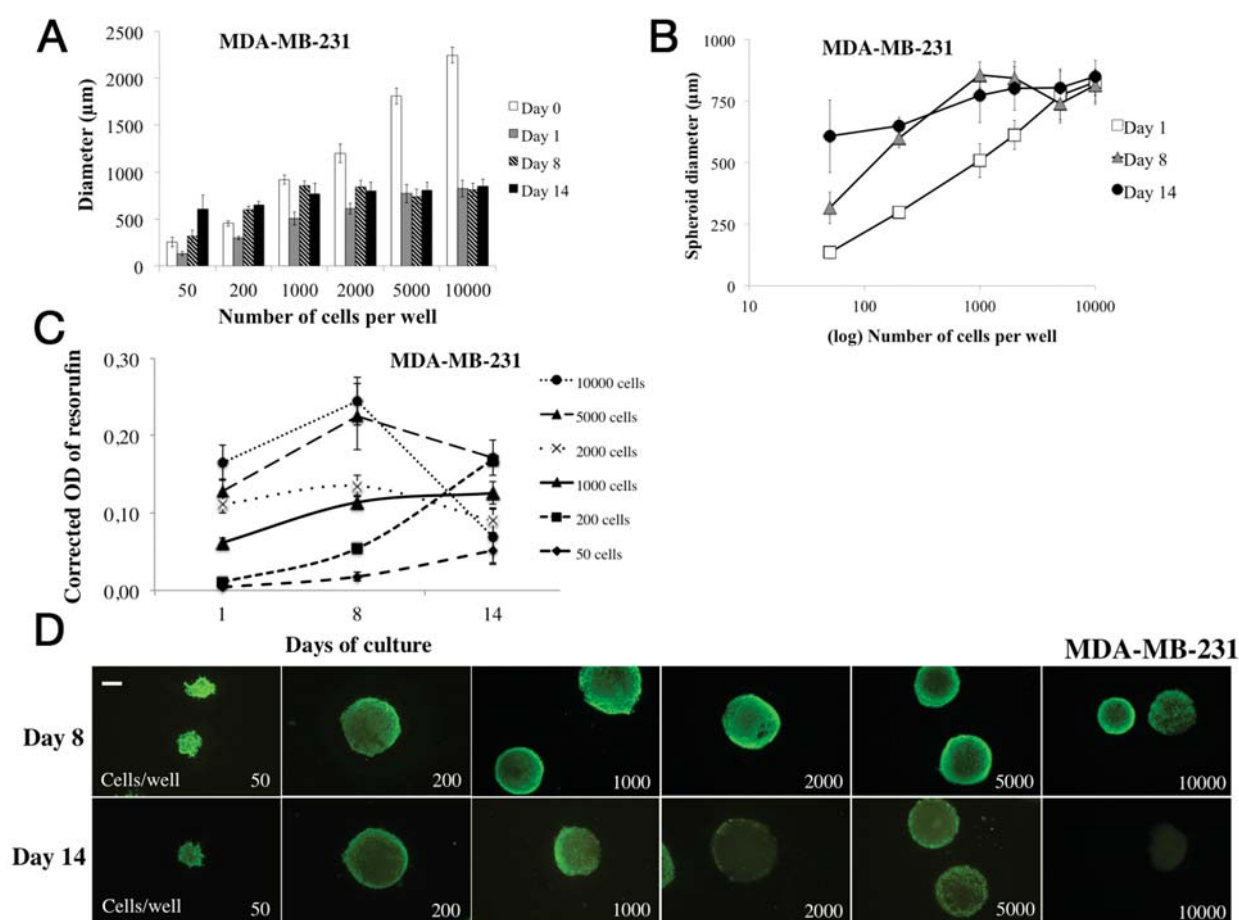


Figure 3: Parameter determination for MDA-MB-231 cell line spheroid formation. For MDA-MB-231 cell line spheroid formation, 50 to 10000 cells were seeded in 96-wells microplates ("ULA" for Ultra Low Attachment, Corning®) at Day 0 and 2% Geltrex® was added to the wells at Day 1. All experiments were carried out at Day 1, Day 8 and Day 14. **(A)** Cell concentration determination for spheroid formation: the diameter of each spheroid was measured by light microscopy with ToupView® software. Day 0 measures correspond to aggregate diameters. **(B)** Spheroid diameter evolution over time: spheroid diameter (μm) was measured with ToupView software® for all tested cell concentrations (log10 scale). **(C)** Cell metabolic activity in spheroids via resazurin test: the resazurin is reduced into resorufin by metabolically active cells. Corrected OD of resorufin ($\lambda 570$ - $\lambda 620 \text{ nm}$) was measured after 15h incubation with $60 \mu\text{M}$ resazurin in PBS. **(D)** Cell viability/mortality in spheroids with Live/Dead® imaging: Green marking corresponds to calcein-AM penetration (viable cells). Red markings correspond to ethidium homodimer-1 cell penetration (dead cells). Scale bar = $200 \mu\text{m}$. Graphs represent at least triplicate biological repeats and are displayed as mean $\pm$ SEM and * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$ and ***** $p<0.00001$.

1 μM ($p<0.00001$), and $66\pm4\%$ vs $54\pm6\%$ with 10 μM ($p<0.00001$), respectively (Figure 7Aa). These results showed a slight decrease in 3D SUM1315 cell viability whatever the cisplatin concentrations. In contrast, viability of 2D cell cultures decreased significantly only with a high cisplatin concentration (10 μM).

SUM1315 spheroid diameters were then measured with ToupView® software. The results showed similar diameters between 0.01 and 0.1 μM cisplatin treated spheroids ($264\pm10\text{ }\mu\text{m}$, $p=0.14$ and $264\pm12\text{ }\mu\text{m}$, $p=0.17$,

respectively) compared to controls ($267\pm11\text{ }\mu\text{m}$) after 5 days of treatment (Figure 7Ab). Nevertheless, a decrease in spheroid diameters was detected after treatment with 1 and 10 μM cisplatin ($223\pm11\text{ }\mu\text{m}$, $p<0.00001$ and $204\pm12\text{ }\mu\text{m}$, $p<0.00001$, respectively). Live/Dead® kit analysis of viability and mortality of 3D cell cultures showed similar cell viability of spheroids treated with 0.01 to 10 μM cisplatin to control spheroids (Figure 7Ac).

For the MDA-MB-231 cell line, analysis of the ratio of the quantity of resorufin formed in DMSO 0.1% treated

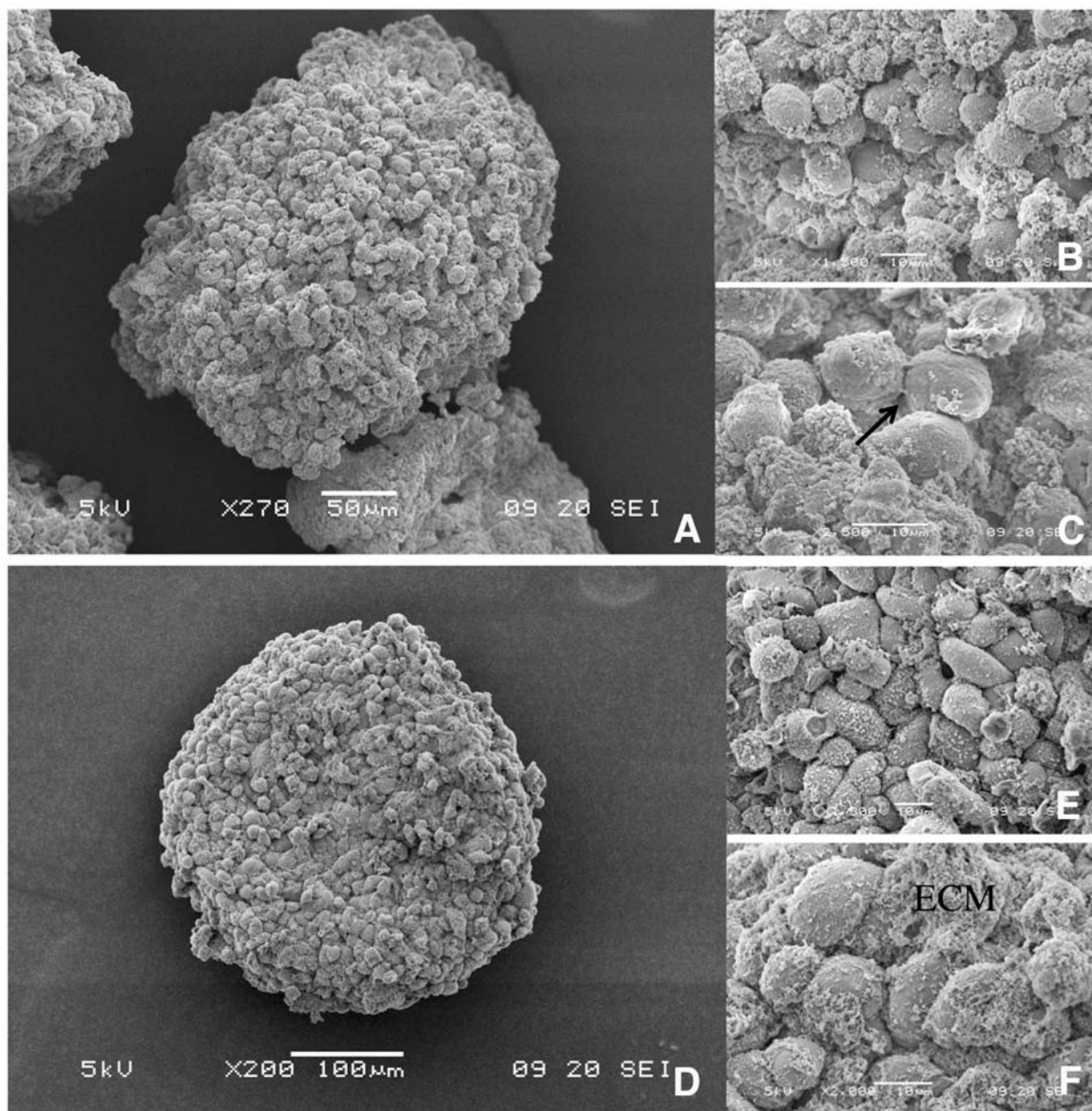


Figure 4: 3D topology of SUM1315 and MDA-MB-231 spheroids by Scanning Electron Microscopy (SEM). Cells were seeded at 1000 cells per well with 2% Geltrex®, added at Day 1. SEM images were taken at Day 5 with Jeol 6060 LV scanning electron microscope. Scale bars are shown on all images. *M*=magnification SUM1315 spheroids: (A) *M*= 270X, (B) *M*=1500X, (C) *M*=2500X MDA-MB-231 spheroids at (D) *M*=200X, (E) *M*=1300X, (F) *M*=2000X. ECM= extracellular matrix, Black arrow= cell junction

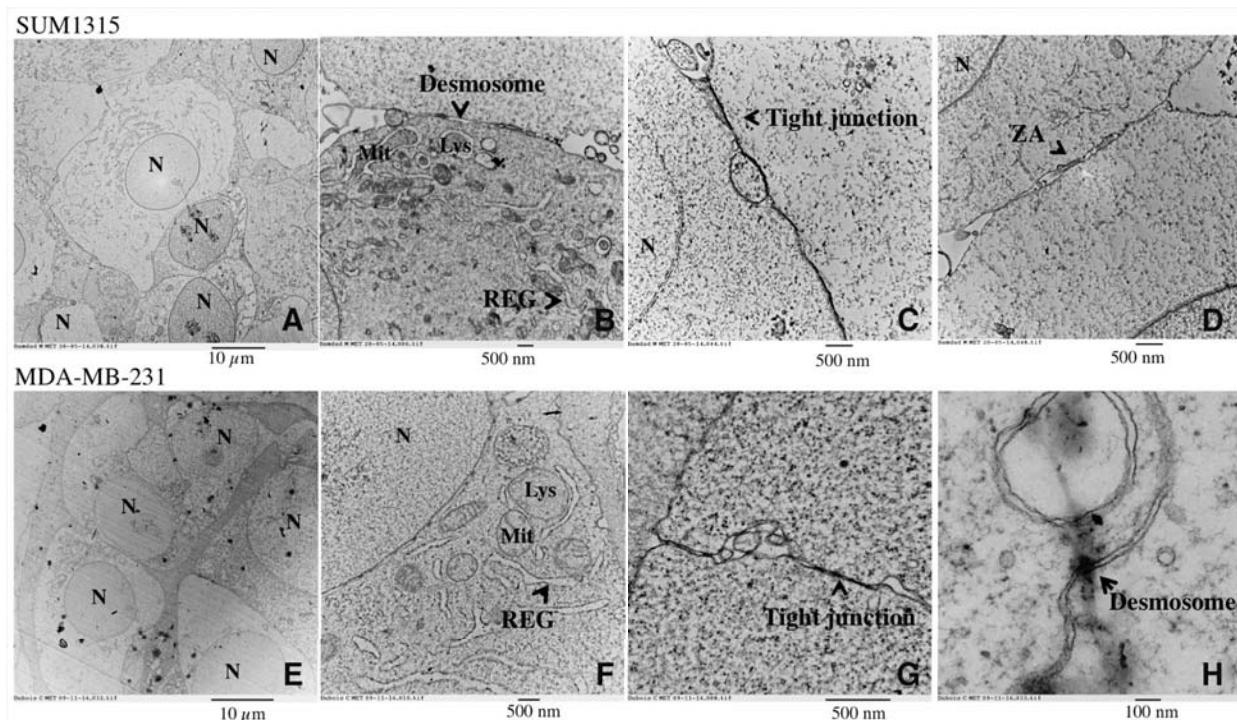


Figure 5: Ultrastructure of SUM1315 and MDA-MB-231 spheroids by Transmission Electron Microscopy (TEM). Cells were seeded at 1000 cells per well with 2% Geltrex®, added at Day 1. Images were taken after 5 days of culture with transmission electron microscope Hitachi H-7650. Scale bars are shown on all images. *M*=magnification SUM1315 cells ultrastructure in 3D cell culture conditions: (A) *M*=3000X, (B) *M*=15000X, (C) *M*=25000X, (D) *M*=25000X. MDA-MB-231 cells ultrastructure in 3D cell culture conditions: (E) *M*=3000X, (F) *M*=20000X, (G) *M*=60000X, (H) *M*=120000X. N=nucleus, Mit=mitochondria, Lys=lysosome, REG= Rough Endoplasmic Reticulum, ZA=zonula adherens. Scale bars are shown on all images.

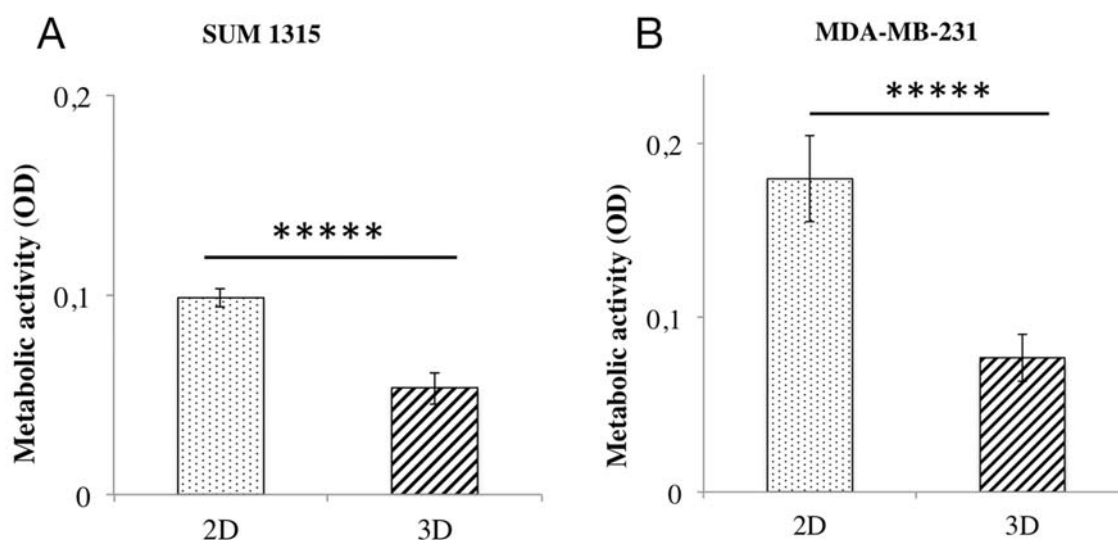


Figure 6: Metabolic activity comparison of 2D vs 3D cell cultures with resazurin test. The resazurin is reduced into resorufin by metabolically active cells. The amount of resorufin formed thus reflects the metabolic activity of the cells. Corrected OD of resorufin (λ 570- λ 620 nm) was measured after 6h of incubation with resazurin in PBS (60 μ M) in the presence of 1000 cells per well at seeding for both cell lines and both culture conditions. Measures were made for both cell lines (A) SUM1315 and (B) MDA-MB-231 and all conditioning regimens after one day of culture. Graphs represent at least triplicate biological repeats and are displayed as mean $\pm$ SEM, ****= p <0.00001.

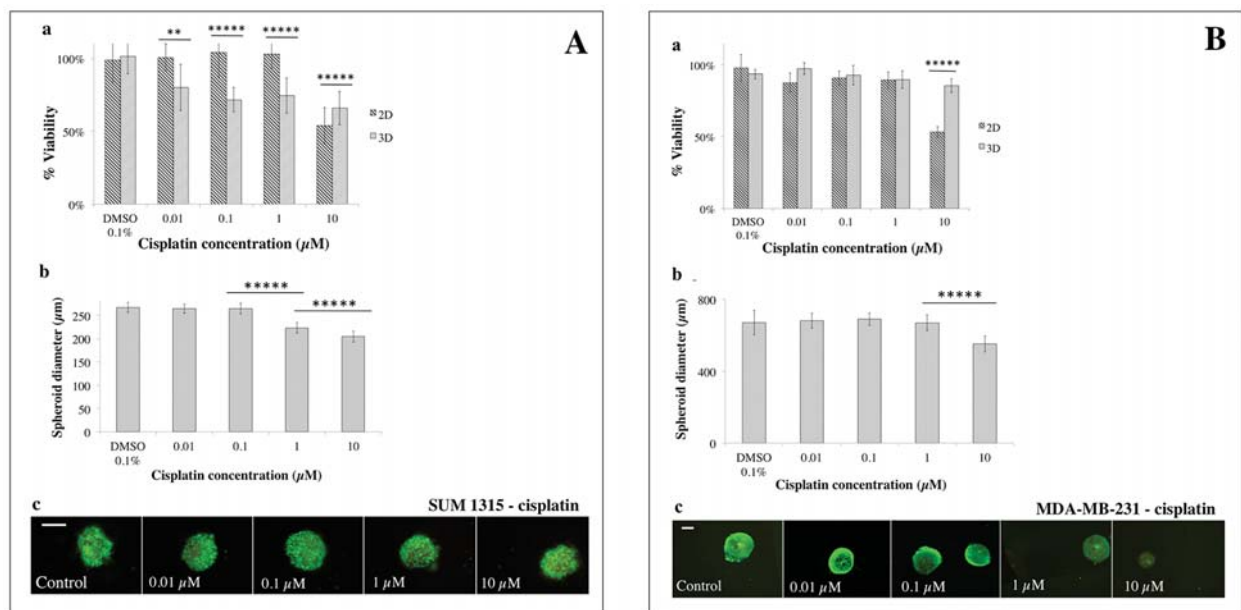


Figure 7: 2D vs 3D cell culture sensitivity to cisplatin. (A) SUM1315 and (B) MDA-MB-231 cell line sensitivity to cisplatin (0.01, 0.1, 1, 10 μM) was assessed in 2D and 3D cell culture conditions after 5 days of treatment. (a) *Cell viability in 2D and 3D culture conditions with the resazurin test*: viability was calculated by the ratio of OD of resorufin formed in cisplatin-treated cells to 0.1% control DMSO cells. (b) *Spheroid diameter measurement*: this parameter was measured with ToupView® software (μm). (c) *Live/Dead® spheroid imaging*: Green marking corresponds to calcein-AM penetration (viable cells). Red markings correspond to ethidium homodimer-1 cell penetration (dead cells). Scale bar = 200 μm . Results are displayed as mean $\pm$ SEM where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ and ***** $p < 0.00001$.

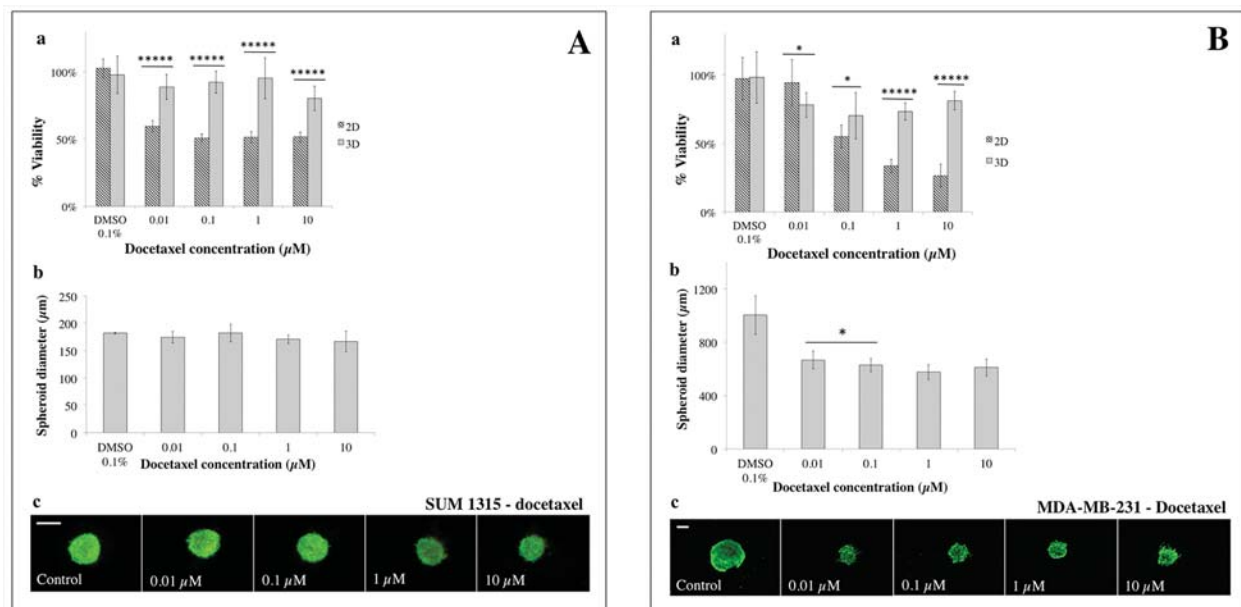


Figure 8: 2D vs 3D cell culture sensitivity to docetaxel. (A) SUM1315 and (B) MDA-MB-231 cell lines sensitivity to docetaxel (0.01, 0.1, 1, 10 μM) was assessed in 2D and 3D cell culture conditions after 5 days of treatment. (a) *Cell viability in 2D and 3D culture conditions with the resazurin test*: viability was calculated by the ratio of OD of resorufin on docetaxel-treated cells to 0.1% control DMSO cells. (b) *Spheroid diameter measurement*: this parameter was measured with ToupView® software (μm). (c) *Live/Dead® spheroid imaging*: Green marking corresponds to calcein-AM penetration (viable cells). Red markings correspond to ethidium homodimer-1 cell penetration (dead cells). Scale bar = 200 μm . Results are displayed as mean $\pm$ SEM where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ and ***** $p < 0.00001$.

cells in comparison with untreated control cells showed no impact of DMSO at this concentration, with $98\pm5\%$ for 2D ($p=0.21$) and $94\pm7\%$ for 3D cell culture ($p=0.16$). With cisplatin, the analysis of 3D and 2D cell viability after treatment showed $97\pm8\%$ vs $88\pm5\%$ viability after $0.01\ \mu\text{M}$ cisplatin ($p<0.01$), $93\pm7\%$ vs $91\pm15\%$ for $0.1\ \mu\text{M}$ ($p=0.68$), $90\pm5\%$ vs $89\pm16\%$ for $1\ \mu\text{M}$ ($p=0.94$) and $86\pm4\%$ vs $53\pm4\%$ for $10\ \mu\text{M}$ ($p<0.00001$), respectively (Figure 7Ba).

These results showed slight cisplatin toxicity with $10\ \mu\text{M}$ on MDA-MB-231 3D cell culture compared to 2D. Moreover, IC_{50} could not be determined for either cell culture.

MDA-MB-231 spheroid diameters analysis at Day 5, in the presence of different concentrations of cisplatin showed similar diameters to controls after treatment with 0.01 to $1\ \mu\text{M}$ cisplatin. Nevertheless, a significant decrease in spheroid size with $10\ \mu\text{M}$ ($551\pm33\ \mu\text{m}$) compared to controls ($680\pm39\ \mu\text{m}$, $p<0.00001$) (Figure 7Bb) was detected. In parallel, similar green markings were observed in spheroids treated with 0.01 to $1\ \mu\text{M}$ cisplatin compared to controls, and lower viability was detected for spheroids treated with $10\ \mu\text{M}$ (Figure 7Bc).

Sensitivity to docetaxel

For the SUM1315 cell line, the analysis of the ratio of the quantity of resorufin formed in DMSO 0.1% treated cells in comparison with untreated control cells showed no

toxicity of DMSO at this concentration, with $102\pm\%$ for 2D ($p=0.27$) and $98\pm14\%$ for 3D cell cultures ($p=0.73$).

SUM1315 cell line sensitivity to docetaxel was assessed under the same culture conditions (Figure 8A). 3D and 2D cell culture viability was $89\pm9.0\%$ vs $58\pm4\%$ with $0.01\ \mu\text{M}$ docetaxel ($p<0.00001$), $92\pm8\%$ vs $49\pm3.0\%$ for $0.1\ \mu\text{M}$ ($p<0.00001$), $95\pm15\%$ vs $50\pm4\%$ for $1\ \mu\text{M}$ ($p<0.00001$) and $80\pm9.0\%$ vs $50\pm4\%$ for $10\ \mu\text{M}$ ($p<0.00001$), respectively (Figure 8Aa). These results showed that sensitivity of the SUM1315 2D cell line to docetaxel remained relatively stable during the experiment in the presence of increasing concentrations of the drug, with the IC_{50} value never reached. The same results were observed for the SUM1315 3D cell line. Nevertheless, as with cisplatin, SUM1315 3D cell culture was less sensitive to docetaxel than the 2D cell culture ($p<0.00001$ for all tested concentrations).

SUM1315 spheroid diameters remained stable in the presence of increasing concentrations of docetaxel ($175\pm11\ \mu\text{m}$, $182\pm16\ \mu\text{m}$, $171\pm8\ \mu\text{m}$, $167\pm19\ \mu\text{m}$ for respectively 0.01 , 0.1 , 1 , $10\ \mu\text{M}$ docetaxel, compared to controls $182\pm1\ \mu\text{m}$ (Figure 8Ab). Live/Dead® kit analysis of viability and mortality of 3D cell cultures showed similar cell viability of treated spheroids to controls for all docetaxel concentrations (Figure 8Ac). These results correlated with the high viability rates observed with the resazurin test for 3D cell culture.

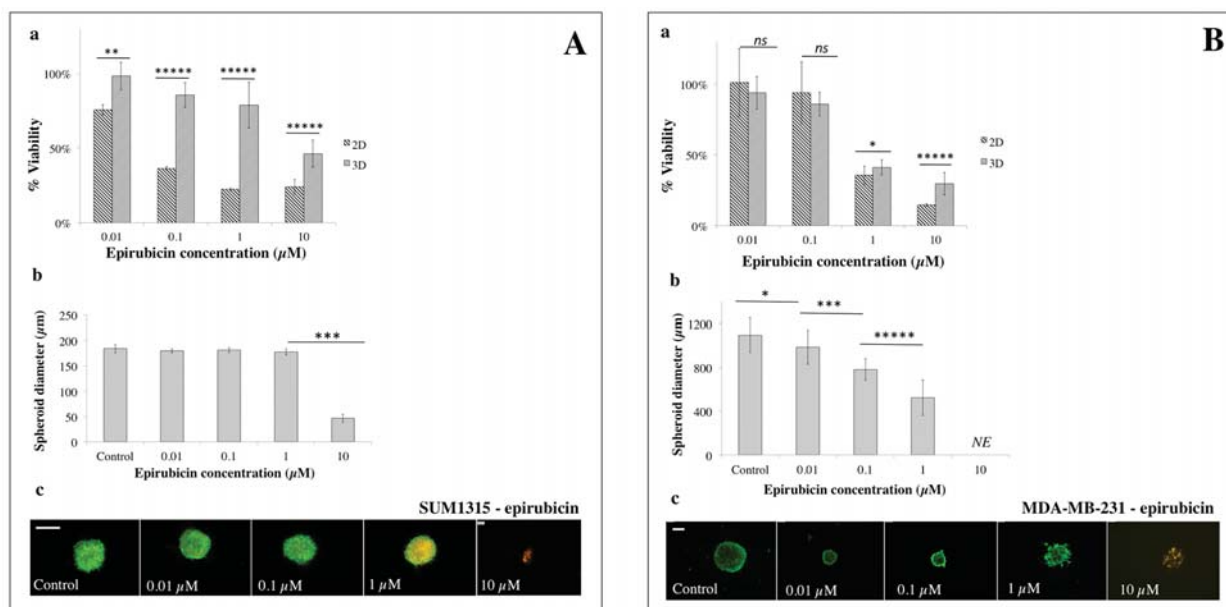


Figure 9: 2D vs 3D cell culture sensitivity to epirubicin. (A) SUM1315 and (B) MDA-MB-231 cell line sensitivity to epirubicin (0.01 , 0.1 , 1 , $10\ \mu\text{M}$) was assessed in 2D and 3D cell culture conditions after 5 days of treatment. (a) Cell viability in 2D and 3D culture conditions with the resazurin test: viability was calculated by the ratio of OD of resorufin in epirubicin-treated cells to 0.1% control DMSO cells. (b) Spheroid diameter measurement: this parameter was measured with ToupView® software (μm). (c) Live/Dead® spheroid imaging: Green marking corresponds to calcein-AM penetration (viable cells). Red markings correspond to ethidium homodimer-1 cell penetration (dead cells). Scale bar = $200\ \mu\text{m}$. Results are displayed as mean $\pm$ SEM where * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$ and ***** $p<0.00001$.

For the MDA-MB-231 cell line, the analysis of the ratio of the quantity of resorufin formed in DMSO 0.1% treated cells in comparison with untreated control cells showed no toxicity of DMSO at this concentration, with $97\pm15\%$ for 2D ($p=0.65$) and $98\pm19\%$ for 3D cell culture ($p=0.80$).

With docetaxel, 3D vs 2D cell culture viability was $78\pm9\%$ vs $94\pm17\%$ for $0.01\text{ }\mu\text{M}$ ($p<0.05$), $70\pm17\%$ vs $55\pm8\%$ for $0.1\text{ }\mu\text{M}$ ($p<0.05$), $73\pm6\%$ vs $33\pm5\%$ for $1\text{ }\mu\text{M}$ ($p<0.00001$), and $81\pm7\%$ vs $27\pm8\%$ for $10\text{ }\mu\text{M}$ ($p<0.00001$), respectively (Figure 8Ba). MDA-MB-231 3D cell culture was again less sensitive than 2D cell culture with IC_{50} values of $0.2\pm0.004\text{ }\mu\text{M}$ for 2D and superior to $10\text{ }\mu\text{M}$ for 3D cell culture, and a ratio 3D/2D > 58 fold.

After docetaxel treatment, MDA-MB-231 spheroid diameters decreased significantly to a plateau with all tested concentrations ($666\pm66\text{ }\mu\text{m}$ with $0.01\text{ }\mu\text{M}$ and $609\pm64\text{ }\mu\text{m}$ with $10\text{ }\mu\text{M}$) in comparison with controls ($1002\pm144\text{ }\mu\text{m}$, $p<0.00001$) (Figure 8Bb). Live/Dead® kit analysis showed that MDA-MB-231 spheroid viability remained stable in the presence of increasing concentrations of docetaxel (Figure 8Bc).

Sensitivity to epirubicin

SUM1315 3D and 2D cell culture viability after epirubicin treatment was $98\pm12\%$ vs $76\pm3\%$ for $0.01\text{ }\mu\text{M}$ ($p<0.001$), $85\pm15\%$ vs $36\pm1\%$ for $0.1\text{ }\mu\text{M}$ ($p<0.00001$), $46\pm4\%$ vs $22\pm1\%$ for $1\text{ }\mu\text{M}$ ($p<0.00001$) and $46\pm4\%$ vs $24\pm5\%$ for $10\text{ }\mu\text{M}$ ($p<0.00001$), respectively (Figure 9Aa). IC_{50} values of 2D and 3D cell culture were $0.1\pm0.004\text{ }\mu\text{M}$ against $7.6\pm0.5\text{ }\mu\text{M}$, respectively. These results showed that 3D cell culture was 140 fold less sensitive to epirubicin than 2D monolayer cell culture.

SUM1315 spheroid diameters remained stable in the presence of 0.01 , 0.1 or $1\text{ }\mu\text{M}$ epirubicin ($180\pm4\text{ }\mu\text{m}$, $181\pm5\text{ }\mu\text{m}$ and $178\pm6\text{ }\mu\text{m}$, respectively) compared to controls ($184\pm8\text{ }\mu\text{m}$) (Figure 9Ab). However, spheroid diameters decreased significantly ($47\pm8\text{ }\mu\text{m}$, $p<0.001$) with $10\text{ }\mu\text{M}$ of the drug. Live/Dead® kit analysis confirmed that SUM1315 spheroids treated with 0.01 and $0.1\text{ }\mu\text{M}$ showed the same viability as controls. In contrast, spheroids treated with $1\text{ }\mu\text{M}$ epirubicin presented a necrotic core (yellow markings), and were dead with $10\text{ }\mu\text{M}$ (red markings) (Figure 9Ac).

For the MDA-MB-231 cell line, 3D and 2D cell culture viability was $94\pm11\%$ vs $101\pm24\%$ for $0.01\text{ }\mu\text{M}$ epirubicin ($p=0.29$), $86\pm8\%$ vs $94\pm22\%$ for $0.1\text{ }\mu\text{M}$ ($p=0.19$), $41\pm5\%$ vs $36\pm7\%$ for $1\text{ }\mu\text{M}$ ($p<0.05$) and $30\pm8\%$ vs $15\pm1\%$ for $10\text{ }\mu\text{M}$ ($p<0.00001$), respectively (Figure 9Ba). IC_{50} values were $0.6\pm0.003\text{ }\mu\text{M}$ for 2D and $0.6\pm0.1\text{ }\mu\text{M}$ for 3D cell culture. These results showed that MDA-MB-231 3D cell culture was as sensitive to epirubicin as 2D cell culture.

MDA-MB-231 spheroid diameters decreased significantly in a dose-dependent manner, in the presence of

increasing concentrations of epirubicin: $986\pm155\text{ }\mu\text{m}$ ($p<0.05$), $782\pm97\text{ }\mu\text{m}$ ($p<0.00001$) and $525\pm163\text{ }\mu\text{m}$ ($p<0.00001$) for 0.01 , 0.1 and $1\text{ }\mu\text{M}$ epirubicin, respectively, against $1096\pm157\text{ }\mu\text{m}$ for untreated spheroids (Figure 9Bb). With $10\text{ }\mu\text{M}$ epirubicin, spheroids were destroyed. Live/Dead kit analysis of viability and mortality showed that for epirubicin concentrations of 0.01 to $1\text{ }\mu\text{M}$, spheroid viability remained stable (Figure 9Bc). In contrast, remaining spheroids treated with $10\text{ }\mu\text{M}$ exhibited a predominance of diffuse red marked cells (Figure 9Bc).

DISCUSSION

To optimize *in vitro* assays for the evaluation of drug sensitivity in TNBL breast cancer cell lines, we have conducted different studies to develop optimal 3D cell cultures from SUM1315 and MDA-MB-231 cell lines, with the “liquid overlay” technique (“Ultra-Low-Attachment” microplates, Corning®). Indeed, this technique presents several benefits, such as a single-per-well spheroid formation, generated naturally by gravity. Furthermore, this method has been validated for a large number of cancer cell lines [18, 19]. The works allowed for determination of optimal culture conditions, metabolic activity, morphology and ultrastructure of cells within the spheroids. After model developing, the impact of several anticancer agents was studied on both 3D cell cultures in comparison to 2D cell cultures sensitivity.

For the development of 3D compact cell mass cultures with SUM1315 and MDA-MB-231 cell lines, the need for an extracellular matrix was first investigated. For this, Geltrex™ LDEV-Free (Reduced Growth Factor Basement Membrane Matrix, GIBCO®) was used as a specific mixture matrix as it contains mainly laminin, collagen IV, entactin and heparin sulfate proteoglycan. With both cell lines, the 2% Geltrex® concentration resulted in the formation of a single compact spheroid per well, with a 3.6 ± 0.3 fold cell compaction for SUM1315 cell line and a 2.1 ± 0.4 fold compaction for the MDA-MB-231 cell line, whatever cell density. Indeed, some cell lines require specific components mimicking the extracellular matrix (ECM) such as laminin or collagen IV, in their culture medium. These components are responsible for the cell/extracellular matrix connections, and are able to generate an environment leading to the formation of intercellular connections [10, 15, 20]. Moreover, the Triple-Negative Basal-Like SUM1315 and MDA-MB-231 cell lines express low protein intercellular junctions such as E-cadherin, which limit the ability of cells to organize themselves spontaneously into compact spheroids [19–22]. Similar Geltrex® concentrations and conditioning regimens were required to form spheroids with MDA-MB-231 in the literature [11, 15].

Cell density for compact and homogenous spheroid formation was then assessed with both cell lines under

the same experimental conditions as described above. Overall results showed different spheroid diameter evolutions according to TNBL cell line, under the same experimental conditions. Indeed, SUM1315 spheroids did not evolve over time in comparison to MDA-MB-231 spheroids which had expanded. This could be explained by a proliferative capacity of the MDA-MB-231 cell line under these experimental conditions in comparison to the SUM1315 cell line that may exhibit a “stem-cell like” phenotype with slowed down proliferation rate [23]. In 3D culture models, cells forming spheroids may or may not proliferate according to cell type and the extracellular matrix environment [24, 25]. Nevertheless, in both TNBL 3D cell cultures, spheroid diameters did not exceed 1000 µm, which could be explained by the fact that the spheroids had reached their growth plateau [19].

The metabolic activity of 3D-non-proliferative SUM1315 and 3D-proliferative MDA-MB-231 spheroids was then measured at day 1, day 8 and day 14 using the colorimetric resazurin test. This test is safe for cells, easily penetrates spheroids and has already been validated for a large number of 3D cell culture models [26, 27]. Overall results showed that SUM1315 spheroids exhibited metabolic activity only until 8 days of culture, whatever the cell density, whereas MDA-MB-231 spheroids exhibited metabolic activity until 14 days of culture with low cell concentrations. The decrease in metabolic activity and/or cell viability with LiveDead® Kit, detected in the presence of high cell concentrations and/or long-term incubation conditions, may be caused by the formation of necrotic or quiescent areas in spheroids, already described in other 3D models [19].

Optimal 3D cell culture conditions with both non-proliferative SUM1315 and proliferative MDA-MB-231 cell lines required adding 2% Geltrex® after aggregate formation in “ULA microplates” (Corning®) in the presence of 1000 cells per well. This conditioning regimen of 3D culture was used to analyze the topology of spheroids with both cell lines by first Scanning Electron Microscopy (SEM). SEM observations showed the homogenous three-dimensional topology of these masses with tightly packed cells at the surface, covered with extracellular matrix. Then, Transmission Electron Microscopy (TEM) observations of “in-depth” spheroid profiles demonstrated that both cell lines resulted in the formation of compact and organized aggregates. Indeed, these spheroids exhibited round shapes with juxtaposed cells presenting intact membranes, intracellular organelles and intercellular junctions [27].

For 3D cell culture development, it was necessary to compare spheroids metabolic activity to 2D monolayer cultures. For both cell lines under the same experimental conditions, metabolic activity of 2D cell culture was significantly higher than that of 3D cell culture, probably due to cellular heterogeneity in spheroids also described in literature [14, 27]. Moreover, the comparison of

metabolic activity between both cell lines in 2D or 3D cell cultures showed systematic lower activity of SUM1315 than MDA-MB-231 cell line, probably correlated to their respective non-proliferative/proliferative profiles.

All these results showed the development of two TNBL 3D cell culture models using the “liquid overlay” technique that allowed the formation of single-per-well spheroids with quick and easy handling, presenting homogenous shapes with high reproducibility. These two 3D non-proliferative and proliferative models were then used to study anticancer agents effectiveness in comparison to 2D cell culture.

For these experiments, three drugs used in treatment protocols for BLTN breast cancers, alone or in combination, such as cisplatin, docetaxel and epirubicin were chosen [28]. Quantitative viability tests, spheroid diameter and Live/Dead® viability tests were performed. Overall results showed that with both non-proliferative SUM1315 and proliferative MDA-MB-231 TNBL cell lines, sensitivity profiles of 3D and 2D cell cultures seemed to be cell line- and drug-dependent. Indeed, cisplatin (platinum salt) is a cell cycle-dependent alkylating agent which creates inter and intrastrand DNA links, inducing serious DNA double-strand damage after the replication fork. Hence, this class of drug is promoted for BLTN tumors in combination with other chemotherapy agents [28]. Similarly, docetaxel is an antimitotic agent from the taxane family which stops cell mitosis division in proliferating tumoral cells. It has shown to be of interest for TNBL tumor treatment when used in combination with anti-angiogenic agents [28]. In our experimental conditions, cisplatin and docetaxel effectiveness analysis showed cytostatic activity only on proliferative MDA-MB-231 cell line whereas only a slight toxicity was detected on non-proliferative SUM1315 cell line model. These results suggest that proliferative-3D-cell-culture models represent a great tool for antimitotic drugs evaluation.

Epirubicin is known to be more effective on triple-negative breast tumors [28]. This drug is a very powerful non cell-cycle dependent DNA intercalating agent from the anthracycline family. With epirubicin, cytotoxicity tests profiles with both cell lines were different. Indeed, this drug showed a dose-dependent cytotoxic activity, whatever cell culture conditioning regimens. Nevertheless, 3D cell culture remained less sensitive to epirubicin than 2D cell culture. These data suggest that the non-proliferative and proliferative-3D-cell-culture models may be adapted to the evaluation of conventional cytotoxic drugs.

All these results demonstrated firstly a difference in response between 2D and 3D cell culture models for the two TNBL cell lines. Indeed, a systematic lowered sensitivity was detected for 3D cell culture compared to monolayer cell culture probably due to 3D cellular heterogeneity and/or resistance phenotypes. This

reproduces partly tumor response *in vivo* [27]. More, according to drug's mechanism of action, *i.e.* cytotoxic or cytostatic, the choice of a proliferative or non-proliferative 3D model seem to be decisive for drug efficacy's prediction. These observations underline the added value of using 3D cell culture as a tool to explore sensitivity and resistance to chemotherapeutics, among the arsenal of models currently used in preclinical studies.

MATERIALS AND METHODS

2D cell culture

BLTN cell lines SUM1315 and MDA-MB-231 were obtained from Asterand. SUM1315 cells were seeded at 25 000 cells per mL in 75 cm² culture dish (Falcon®) at 37°C under 5% CO₂, in 15 mL Ham's F12 medium (Gibco®) supplemented with 5% decompartmented fetal calf serum, 10 mM HEPES* buffer, 20 mg/mL gentamycin, 10 ng/mL EGF and 4 µg/mL of insulin, according to the supplier's instructions [29, 30]. The MDA-MB-231 cell line was seeded at 25 000 cells per mL in 75 cm² culture dish (Falcon ®) at 37°C under 5% CO₂, in 15 mL RPMI 1640 medium (Gibco®) supplemented with 10% decompartmented fetal calf serum and 20 mg/mL gentamycin, according to the supplier's instructions [29, 30].

Cells having reached confluence were washed with Phosphate Buffer Saline (PBS, 1X, Sigma®) and trypsinized (Trypsin 1X, Sigma®) for 5 min at 37°C. Cells were taken up in 12 mL of culture medium and centrifuged for 10 minutes at 250 G. The pellet was taken up in 5 mL of culture medium and the number of cells per mL was determined by a cell count using a vital stain Trypan Blue. This allowed for counting the cell dilutions necessary for seeding cells in varying concentrations and defined for each experiment.

Liquid overlay 3D cell culture

Cells were seeded in "Ultra-Low-Attachment" round bottom microplates (Corning®) in order to prevent cellular adhesion to the support. After 24h of incubation, Geltrex® was deposited on aggregated cells, and microplates were agitated on a microplate shaker at 185 rpm for 20 min. Spheroid formation and diameter measurements were assessed with a light microscope (CFM-BDS-200 Realux Cofemo) associated with a camera (Industrial Digital Camera, UCMOS05100KPA) and ToupView® software.

Metabolism activity assessment using the resazurin test

The resazurin test is a colorimetric assay for cell viability that involves the reduction of resazurin to resorufin by the metabolically active mitochondria of

cells. Resorufin is a fluorescent compound that exhibits a pink colouring. After a time of incubation, the amount of resorufin formed is directly proportional to the number of metabolically active cells. Cells cultured in 2D or 3D treated with the drugs for 5 days at 37°C were washed with PBS* and incubated with 60 µM of resazurin solubilized in PBS. Optical densities were measured at 570 and 620 nm after 6 to 15 hours of incubation. The amount of reduced compound (resorufin) corresponding to the number of active cells per well was calculated for each condition. The survival rate was determined considering the ratio $(OD_{570-620nm} \text{ treated well} / OD_{570-620nm} \text{ untreated control}) \times 100$. The half maximal inhibitory concentration (I_{C50}) was calculated with the following formula: $I_{C50} = EXP(LN(\text{concentration} > 50\% \text{ inhibition}) - ((\text{signal} > 50\% \text{ inhibition} - 50) / (\text{signal} > 50\% \text{ inhibition} - \text{signal} < 50\% \text{ inhibition})) * LN(\text{concentration} > 50\% \text{ inhibition} / \text{concentration} < 50\% \text{ inhibition}))$.

Imaging of 3D cell culture with the Live/Dead® kit

The Live/Dead® kit (Viability/Cytotoxicity kit, Molecular Probes™) is composed of two fluorochromes, the calcein-AM (green fluorescence) which is retained in viable cells and the ethidium homodimer-1 (ethD1, red fluorescence) which penetrates and binds to DNA of cells in phase of apoptosis or necrosis. The two probes were diluted in phosphate saline buffer to final concentrations of 4 mM for Calcein-AM and 2 mM for ethD-1. After several washings in Phosphate Buffer Saline (PBS), spheroids were incubated with 100 µL of the Calcein-AM/EthD-1 solution for at least 45 min., protected from light. Observations were made with an epifluorescence microscope (Leica DMI 3000B, FITC canal) associated with CCD camera and Leica® Software.

Scanning electron microscopy

Scanning Electron Microscopy (SEM) assessed organization of cell components of the spheroids. Spheroids were washed with 0.2 M sodium cacodylate buffer pH 7.4 and fixed in 1.6% glutaraldehyde in cacodylate buffer overnight at 4°C. They were then rinsed in the same buffer and post-fixed for 1h with osmic acid in cacodylate buffer at ambient temperature. For spheroid visualization, a solution of 0.15% ruthenium red in water was added to the washes and fixative solutions. Fixed spheroids were then progressively dehydrated using a graded ethanol series (10 min. each in 25%, 50%, 70%, 95%, 100% ethanol) and hexamethyldisilazane (3x10 min.). After drying, the samples were mounted on stubs using adhesive carbon tabs and sputter-coated with gold-palladium (JFC-1300, Jeol). Spheroids were observed with a scanning electron microscope Jeol 6060-LV at 5 kV in high-vacuum mode.

Transmission electron microscopy

Transmission Electron Microscopy (TEM) achieved ultrastructural observations of spheroids. Spheroids were washed in 0.2M sodium cacodylate buffer (pH7.4), fixed in 1.6% glutaraldehyde for 24 hours at 4°C. Specimens were then washed three times (10 min.) in Na cacodylate buffer, post fixed 1h with 1% osmium tetroxide in Na cacodylate buffer and washed three times in Na cacodylate buffer. In order to visualize the spheroids, a solution of 0.15% ruthenium red in water was added to the buffer and fixative solutions. Fixed spheroids were then progressively dehydrated in ethanol (70%, 95%, 100%) and acetone. Subsequently, they were infiltrated with acetone and EPON resin mixture (2:1, 1:1, 1:2 for 1h). Specimens were embedded in resin EPON overnight at room temperature, and cured 2 days at 60°C. Thin sections (70 nm) were cut using a UC6 ultramicrotome (Leica) and stained with uranyl acetate and Pb citrate. Carbon was evaporated using CE6500 unit. Observation of the ultrastructural organization of cells within the spheroids was then conducted with a transmission electron microscope (Hitachi H-7650) at 80 kV acceleration voltage. Micrographs were made using a Hamamatsu AMT camera placed in a side position.

Drug solubilization and cell exposure

Docetaxel, cisplatin and epirubicin were prepared at a stock solution of 10 mM. Cisplatin and docetaxel were not soluble in water. These were solubilized in dimethylsulfoxide (DMSO) and the final concentration in each cell culture well was always of 0.1%. Epirubicin was solubilized in distilled water. For all drug treatment experiments, cells cultured in 2D and 3D condition were seeded at a concentration of 1000 cells per well for both cell lines. Cells were then exposed to the drugs 24h after seeding for 2D cell culture (phase of exponential growth) and 24h after Geltrex® adding for 3D cell culture. After five days of incubation, cell viability was then assessed for each condition with the resazurin test. For 3D cell culture, spheroid diameter measurements and Live/Dead® viability/mortality tests were also performed at the end of each treatment.

Statistical analysis

Results were expressed as means $\pm$ standard deviation of n independent experiments. All experiments were performed at least in triplicate and then statistically compared using a Student's t-test. Tests were two-sided and the nominal level of significance was $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****) and $p < 0.00001$ (*****).

Abbreviations

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

PBS: phosphate buffered saline

Author contributions

C.D., R.D., M.B., P.D., E.M., C.A., F.P.L. conceived and designed the study; C.D., R.D., C.B., C.S. performed the experimental work; C.D., R.D., P.D., C.B. E.M. and M.B. analyzed the data; C.D. performed statistical analysis; C.D. R.D., C.B. and M.B. wrote the manuscript. All authors reviewed the manuscript.

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CONFLICTS OF INTEREST

The authors declare no competing financial interests.

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III) Conclusion sur les travaux

Afin d'optimiser les modèles permettant d'évaluer l'efficacité des molécules dans les tests précliniques, les cultures cellulaires en trois dimensions ont été développées avec succès à partir des deux lignées cellulaires MDA-MB-231 et SUM1315. En outre, le suivi de la prolifération et de l'activité métabolique des sphéroïdes a mis en évidence des profils de prolifération cellulaires différents : un modèle prolifératif pour la lignée MDA-MB-231 et un modèle non prolifératif pour la lignée SUM1315. Par la suite, l'ensemble des tests de toxicité réalisés à partir de ces modèles a permis de démontrer l'importance d'un modèle de sphéroïdes prolifératif ou non prolifératif pour la mise en évidence de l'activité (i) cytotoxique et/ou (ii) cytostatique des médicaments. Ainsi, le choix d'un modèle de sphéroïde adapté semble être déterminant pour démontrer l'efficacité des médicaments développés. L'ensemble de ces expériences souligne la valeur ajoutée de la culture cellulaire 3D comme outil de diagnostic permettant de définir la sensibilité et/ou la résistance à la chimiothérapie, dans les études précliniques.

Afin d'optimiser les conditions de culture des sphéroïdes en termes de reproductibilité et d'homogénéité, la deuxième partie des travaux de thèse s'est orientée sur la caractérisation et l'adaptation d'un milieu synthétique sans sérum (**OptiPASS**).

B) Adaptation d'un milieu sans sérum pour la culture cellulaire de deux lignées TN en deux et trois dimensions (2D et 3D).

I) Introduction

L'ensemble des travaux de développement des sphéroïdes TN a été réalisé dans leur milieu de culture d'origine contenant des suppléments d'origine animale, tel que le sérum de veau fœtal. Ce dernier permet un apport en protéines et en facteurs de croissance qui sont essentiels aux cultures cellulaires 2D pour leur maintien, leur attachement et leur prolifération. Néanmoins, la forte variabilité entre les lots de sérum ainsi que leur potentielle contamination par des mycoplasmes induisent un risque élevé de modification du « comportement cellulaire » et par conséquent, un manque de reproductibilité entre les expériences. Afin de palier à ces freins, des milieux de culture sans sérum sont de plus en plus développés.

Afin d'optimiser la réponse prédictive des modèles de sphéroïdes TN développés en conditions normalisées, nous avons développé les cultures cellulaires en 2D et 3D de lignées tumorales mammaires TN dans un nouveau milieu synthétique appelé **OptiPASS** (Société Biopass). Ce dernier a été développé à l'origine pour la croissance et la différenciation des cellules souches mésenchymateuses. Pour ce faire, différents paramètres ont été analysés et comparés au milieu de référence tels que l'adhérence, la prolifération et le maintien du phénotype cellulaire en condition de culture 2D. Par la suite, la prolifération, le profil de viabilité/mortalité ainsi que la sensibilité à l'épirubicine ont été déterminés sur les modèles de sphéroïdes obtenus à partir du milieu **OptiPASS**.

Ces travaux ont fait l'objet d'une publication en premier auteur soumise dans la revue Experimental Cell Research (IF= 3,3)

II) (Article rédigé) The new serum-free medium OptiPASS, an adapted tool for the 3D cell culture of both MDA-MB-231 and SUM1315 Triple-Negative Breast Cancer cell lines.

Three-dimensional culture development of MDA-MB-231 and SUM1315 Triple-Negative Breast Cancer cell lines in the new synthetic serum-free medium OptiPASS.

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ABSTRACT

Triple Negative breast cancers are particularly aggressive and of poor prognosis. *In vitro* cultures are one of the major pathways for developing anticancer strategies and screening targeted therapies. The effectiveness and reproducibility of drug screenings depend largely on the composition and homogeneity of culture media. In order to optimize the predictive responses of triple negative breast cancer 3D cell culture models, these works were focused on the study of SUM1315 and MDA-MB-231 cell lines response in OptiPASS medium, a new serum-free formulation (BIOPASS). In monolayer cell culture condition, cell adherence, proliferation, viability and phenotype were maintained in OptiPASS medium, for MDA-MB-231 cell line. For SUM1315 cell line, these parameters were less efficient due to the lack of cellular adhesion, but led to satisfying proliferation rates, allowing the production of spheroids. For 3D cell cultures produced in OptiPASS, spheroid size monitoring showed a 1.3 and 1.5-fold increase for MDA-MB-231 and SUM1315 cell lines, respectively. Viability and mortality profiles were also maintained in OptiPASS. Moreover, drug sensitivity thresholds were improved in 3D cell cultures allowing the realization of quicker high throughput drug screenings. All these experiments showed that OptiPASS is suitable for 2D and 3D cell cultures of these two Triple-

Negative breast cancer cell lines, with a reproducibility of spheroid formation superior to 98%. That opens the way to the common use of this synthetic medium in future preclinical breast cancer research studies.

Keywords: Synthetic medium OptiPASS, Triple Negative Breast Cancer cell lines, spheroid, proliferative 3D cell culture, drug screening.

INTRODUCTION

Triple-Negative Breast Cancers (TNBC) are particularly aggressive and of poor prognosis ^{1,2}. It is a heterogeneous and complex pathology, characterized by the non-expression of estrogen receptors (ER) and progesterone receptors (PR), and the absence of Epidermal Growth Factor Receptor-2 (HER2) over-expression or ERBB-2 amplification ^{3,4}. Recently, five molecular subtypes of TNBC tumours have been described by Lehmann⁵ which are (i) basal-1, (ii) basal-2, (iii) mesenchymal-like, (iv) androgen-receptor and (v) immunomodulatory. Currently, therapies specifically targeting the characteristics of these tumors are under development in preclinical and clinical studies ^{6,7}. In order to model the effectiveness of these targeted therapies, laboratories performing preclinical studies use *in vitro* cell culture techniques. This methodology allows the reproduction of cell physiological conditions. As a result, it must respect certain parameters essential to cell survival such as pH, temperature, osmolarity and oxygen supply. In order to control these parameters, specific culture media have therefore been developed, allowing cell survival and proliferation ⁸. The culture medium is one of the key factors of *in vitro* modelling. Thus, there are different categories of culture media, with common characteristics and variants, depending on the type of cells studied ⁹. The majority of the cell culture media are supplemented with fetal calf serum (FCS), which is a supplement of animal origin. It is required for culture of mammalian cells as it provides multiple elements essential to initial adhesion, growth, metabolism and proliferation of cells in the culture ⁸. Its composition is complex because of its biological origin, but its main constituents are growth factors such as insulin, transport proteins such as albumin, attachment factors promoting cell adhesion such as fibronectin, and amino acids. It also contains vitamins such as vitamin B, some essential minerals, lipids, fatty acids and protease inhibitors, which possess an action against proteolytic enzymes such as trypsin. FCS also acts as a buffer to maintain pH and protects the cells from shocks, thanks to its viscosity ¹⁰. Despite its many advantages, the use of the FCS presents several drawbacks. Indeed, like any compound of biological origin, its general composition is not controllable, resulting in a variability between batches. This variability requires the systematic control of batches' quality in terms of cell

growth performance and the absence of contaminants (mycoplasma, viruses, etc.) or growth inhibitors. Furthermore, the biological origin of this reagent poses ethical issues, particularly with regard to animal protection and suffering, as well as problems of stock depletion leading to shortages ¹¹. Thus, the contribution of the FCS to culture media seems necessary, but also controversial. For these reasons, the common use of serum-free synthetic media would enhance the homogeneity of experiments by increasing the level of standardization, thus improving the quality of cell modelling worldwide ¹². This discipline is using more and more three-dimensional (3D) cell culture models, as they reproduce more precisely the tumor microenvironment. Indeed, 3D cell cultures produced by liquid overlay or scaffold techniques, allow the formation of pseudo-microtissues also called spheroids or organoids ^{13,14}. These structures are able to produce their own cell-to-cell and cell-to-matrix interactions, leading to interesting genetical, biochemical and phenotypical gradients. These methods are long to develop, but extremely advantageous, especially for Oncology preclinical modeling. The mastering of their reproducibility in terms of growth characteristics is essential for efficient drug screening and efficacy prediction ¹⁴⁻¹⁷.

For these research works, two liquid-overlay 3D cell culture types already developed by our team for drug screening (i) the proliferative MDA-MB-231 spheroid model and (ii) the non-proliferative SUM1315 spheroid model were used. This modeling highlighted that the choice of a proliferative or non-proliferative 3D model seems to be decisive for drug efficacy's prediction, according to drug's mechanism of action, *i.e.* cytotoxic or cytostatic¹⁸. In this context, to optimize the predictive responses of these both 3D cell culture models to several chemotherapeutics, these works were focused on the study of SUM1315 and MDA-MB-231 cell lines response in a new synthetic serum-free medium, OptiPASS (BIOPASS). For this, several cell parameters such as morphology, metabolic activity, viability/mortality and sensitivity to anticancer drug were analyzed in 2D or 3D cell culture conditions with OptiPASS medium and with their reference-serum-supplemented media.

A

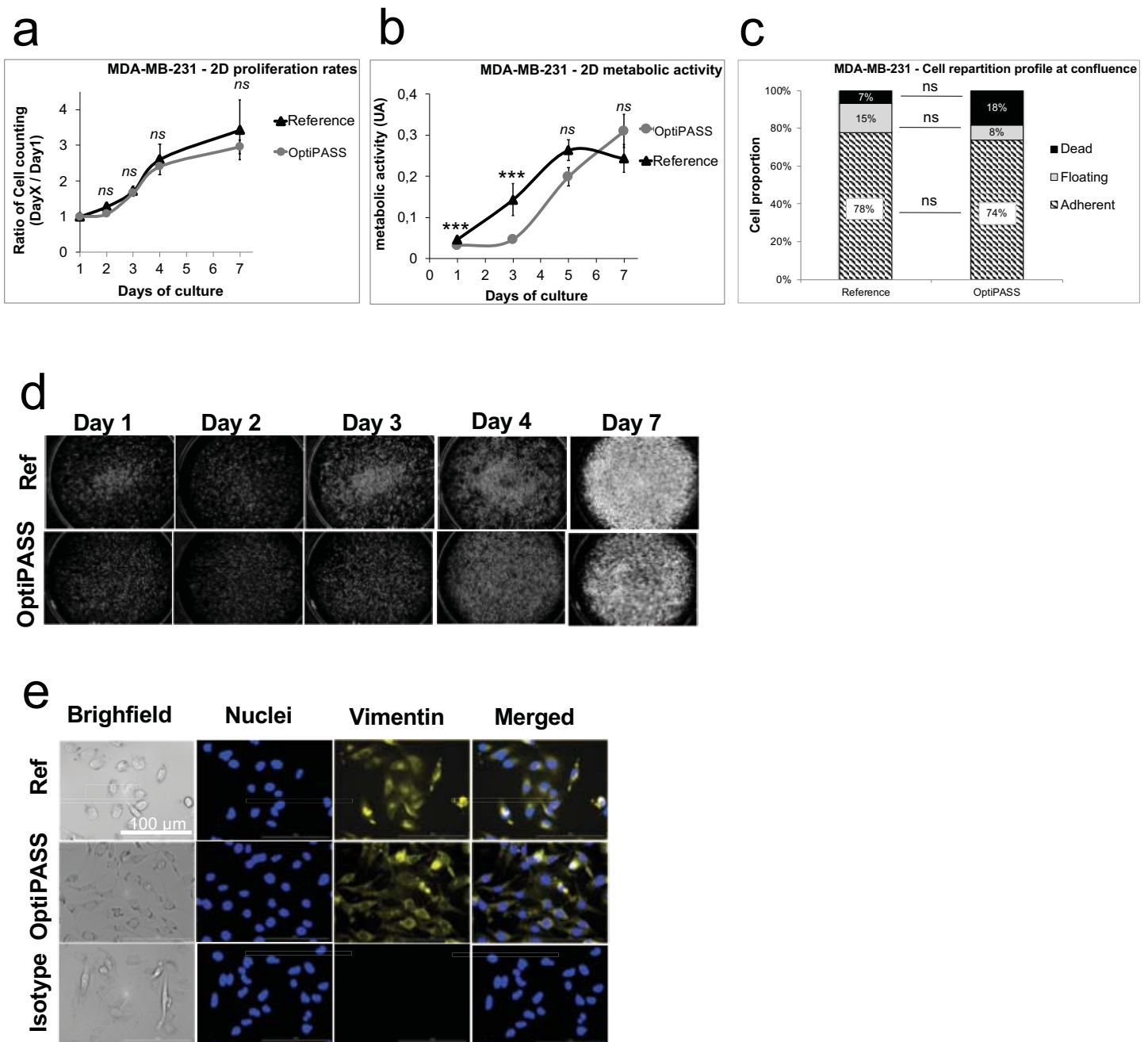


Fig 1A. MDA-MB-231 monolayer cell culture development with OptiPASS medium. **a: 2D proliferation rates:** the number of adherent cells was counted with Cytation3MV plate reader (BIOTEK) after 1, 2, 3, 4 and 7 days of culture and normalized to cell counting at day 1, $n=9$, bars=s.e.m. **b: Cell metabolic activity monitoring:** the metabolic activity of adherent cells was quantified with resazurin test for 7 days and normalized to cell metabolic activity quantified at day 1, $n=16$, bars=s.d.. **c: Cell repartition profile:** cells cultured in reference medium or OptiPASS medium were harvested and counted with a blue trypan exclusion test. Three cell categories are represented: “floating” and “dead” cells were counted in the supernatant medium, and “adherent” cells were counted after trypsinisation of the cellular carpet. $n=5$, ns= non significant difference with student t-test **d: Cellular carpet morphology in phase contrast imaging:** each well of the 6-well microplate was imaged at day 1, 2, 3, 4 and 7 (confluence) with Cytation3MV plate reader. Images (M=4X) were stitched to allow an overview of the all well. Well area = 9.6 cm². **e: Vimentin immunofluorescent staining:** cells cultured in reference or OptiPASS medium were immunoassayed for vimentin expression with Cytation3MV (GFP and Cy5 filters). The isotype control is the negative control for vimentin expression. Scale bar = 100 μ m, blue=nuclei, yellow=vimentin, ns = non-significantly different, ***** = $p<0.00001$, two-sided student's t-test comparison.

RESULTS

1. Monolayer (2D) culture development of TNBC cell lines with OptiPASS medium

In this study, the performances of the serum-free medium OptiPASS were first determined in 2D cell culture on two TNBC cell lines, MDA-MB-231 and SUM1315. For this, cells were cultured with their reference medium (containing 5 to 10% of FCS, according to cell line) or OptiPASS medium (coated with 2% Geltrex), until confluence. In order to characterize cell behavior, several parameters such as (i) cell adherence, (ii) cell proliferation, (iii) cell metabolic activity and (iv) cell morphology were analyzed in both cell culture media. Moreover, the mesenchymal phenotype status of both cell lines was also determined in each culture medium by a vimentin immunostaining (Fig 1).

For MDA-MB-231 cell line, the cell proliferation rate was first studied in both culture media by a cell counting experiment using the Cytation3MV plate reader. For this, the ratio of adherent cells at day X on adherent cells at day 1 (one day after seeding) was calculated (Fig 1Aa). The proliferation level increased from 1.7 ± 0.1 and 1.7 ± 0.1 at day 3 ($p=0.71$, two-sided student's t-test), to 3.4 ± 0.8 and 3.0 ± 0.2 at day 7 ($p=0.05$) for reference medium and OptiPASS medium, respectively. The cell proliferation time course seemed to be similar in reference and OptiPASS medium. Parallely, cell metabolic activity was monitored for the 7 days of culture by the resazurin test. It was 0.181 ± 0.039 and 0.084 ± 0.006 at day 3 ($p=0.0005$) and increased to 0.335 ± 0.078 and 0.366 ± 0.056 at day 7 ($p=0.40$), for reference and OptiPASS medium, respectively (Fig. 1Ab). Then, the cell repartition profiles (either adherent to the support, floating in the supernatant or dead in the supernatant) were analyzed for both cell culture conditions with a blue trypan exclusion test at confluence (at day 7) for reference and OptiPASS medium (Fig 1Ac). The proportions of MDA-MB-231 cells attached to the support in reference and OptiPASS medium were similar with $78 \pm 8\%$ and $74 \pm 11\%$ ($p=0.64$), respectively. Furthermore, the proportions of floating-living cells in reference medium were not significantly different to OptiPASS medium. Indeed, it was of $15 \pm 9\%$ in reference medium and of $8 \pm 5\%$ ($p=0.30$) in OptiPASS medium. Finally, the rate of dead cells in reference medium was of $7 \pm 3\%$ at confluence and similar to OptiPASS with $18 \pm 11\%$ ($p=0.20$). These results showed that the proportions of “adherent”, “floating” and “dead” cells in OptiPASS medium were similar to reference medium. Thus, the morphology of the cellular carpet observed in digital phase contrast for the seven days of culture showed no difference between cells cultured in

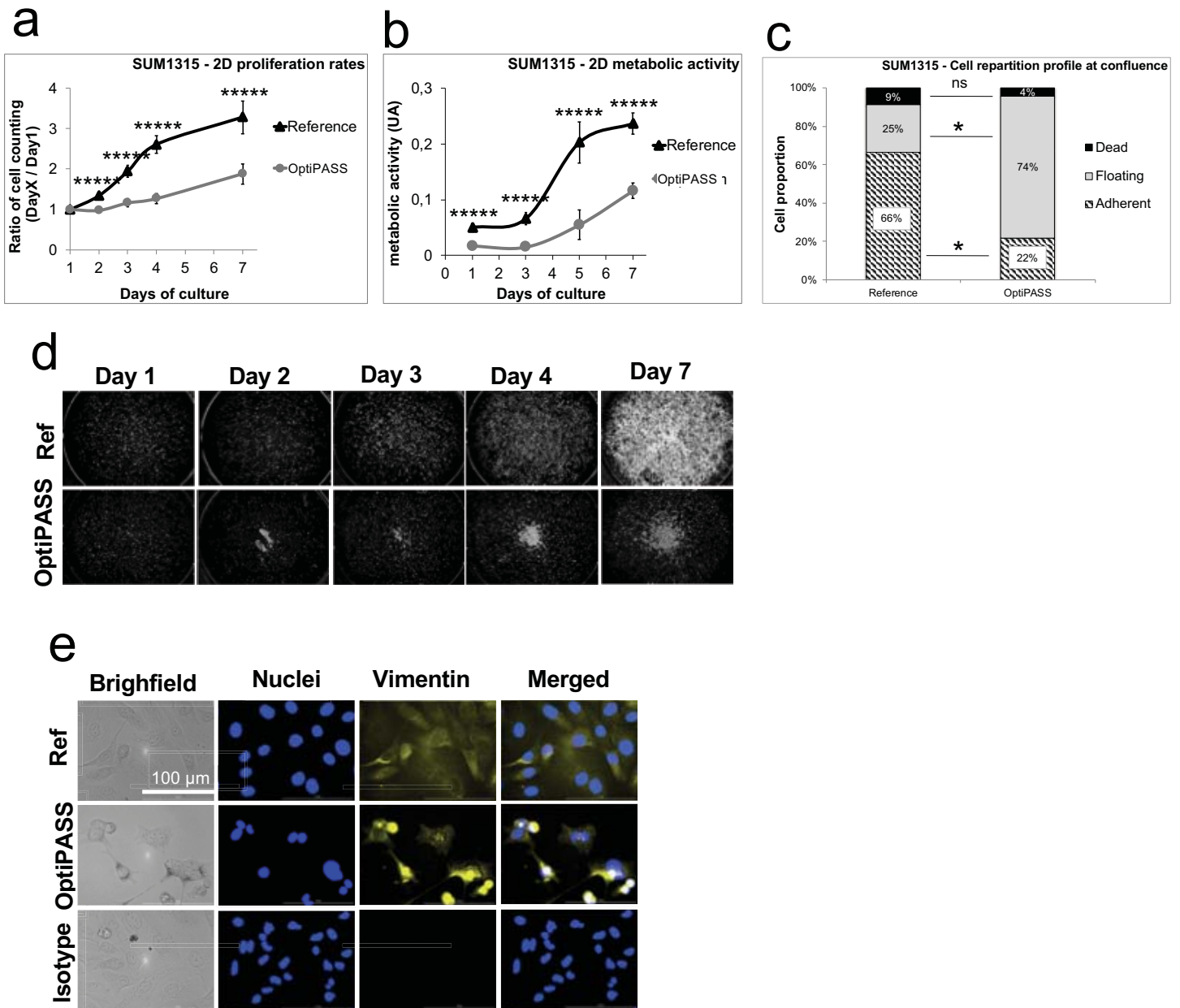
B

Fig 1B. SUM1315 monolayer cell culture development with OptiPASS medium. **a: 2D proliferation rates:** the number of adherent cells was counted with Cytation3MV plate reader (BIOTEK) after 1, 2, 3, 4 and 7 days of culture and normalized to cell counting at day 1, $n=9$, bars=s.e.m. **b: Cell metabolic activity monitoring:** the metabolic activity of adherent cells was quantified with resazurin test for 7 days and normalized to cell metabolic activity quantified at day 1, $n=16$, bars=s.d.. **c: Cell repartition profile:** cells cultured in reference medium or OptiPASS medium were harvested and counted with a blue trypan exclusion test. Three cell categories are represented: “floating” and “dead” cells were counted in the supernatant medium, and “adherent” cells were counted after trypsinisation of the cellular carpet. $n=5$, ns= non significant difference with student t-test **d: Cellular carpet morphology in phase contrast imaging:** each well of the 6-well microplate was imaged at day 1, 2, 3, 4 and 7 (confluence) with Cytation3MV plate reader. Images (M=4X) were stitched to allow an overview of the all well. Well area = 9.6 cm^2 . **e: Vimentin immunofluorescent staining:** cells cultured in reference or OptiPASS medium were immunoassayed for vimentin expression with Cytation3MV (GFP and Cy5 filters). The isotype control is the negative control for vimentin expression. Scale bar = $100 \mu\text{m}$, blue=nuclei, yellow=vimentin, ns = non-significantly different, ***** = $p<0.00001$, two-sided student’s t-test comparison.

OptiPASS medium compared to reference medium (Fig 1Ad). Finally, vimentin immunostainings analysis carried out on MDA-MB-231 cell line (Fig 1Ae) showed the constant expression of the mesenchymal marker in cells cultured with both culture media. These results demonstrated that for all analyzed parameters *ie*, cell proliferation rates, cell metabolic activity and the proportion of attached cells/floating/dead cells, similar cell culture performances were detected for OptiPASS and reference media, with MDA-MB-231 cell line.

For SUM1315 cell line, the cell proliferation analysis showed growth rates of 1.9 ± 0.2 at day 3, 3.3 ± 0.4 at day 7 in reference medium and of 1.2 ± 0.1 at day 3 ($p=10^{-6}$ compared to reference) and 1.9 ± 0.3 at day 7 ($p=10^{-12}$ compared to reference), in OptiPASS medium (Fig 1Ba), respectively. Similarly, the cell metabolic activity was determined in the same experimental conditions and was of 0.217 ± 0.016 in reference medium and 0.057 ± 0.004 in OptiPASS medium ($p=10^{-9}$) at day 3. Then, it increased at day 7 for both cell culture media with 0.148 ± 0.019 and 0.067 ± 0.014 ($p=10^{-6}$), for reference and OptiPASS, respectively (Fig 1Bb). Then, the proportion of adherent cells, living-floating cells and dead-floating cells was analyzed in each cell culture medium at confluence (Fig 1Bc). The proportion of adherent cells in reference medium was of $66 \pm 12\%$. In contrast, with OptiPASS medium, it was lower with $22 \pm 14\%$ ($p=0.01$ compared to reference) (Fig 1Bc). Conversely, the rate of floating-living cells remained lower in reference medium with $25 \pm 10\%$ compared to $74 \pm 15\%$ in OptiPASS ($p=0.01$) (Fig 1Bd). Interestingly, no significant difference in the rate of dead cells was detected between reference and OptiPASS medium with $9 \pm 7\%$ and $4 \pm 1\%$ ($p=0.37$), respectively (Fig 1Bc). Then, the observations of cell morphology in digital phase contrast (Fig 1Bd) showed a majority of homogeneously spread and attached cells in reference medium. In contrast, a cluster of round-detached cells in the middle of the wells was detected with OptiPASS medium. Nevertheless, the immunostaining experiment confirmed the expression of the vimentin marker with both cell culture conditions (Fig 1Be), demonstrating that SUM1315 cells cultured in OptiPASS medium had kept their mesenchymal phenotype. For SUM1315 cell line, all studied parameters showed a greater performance in terms of cell proliferation and cell metabolic activity with reference medium compared to OptiPASS medium. More, similar cell repartition profiles were detected by the time (from seeding to confluence), for each culture medium. However, the cellular behavior of SUM1315 cell line was different in OptiPASS medium. Indeed, in this latter, a higher proportion of living-floating cells was detected suggesting that despite the weak attachment of cells in this medium, they remained alive over time. Similarly, the cell proliferation rate of adherent cells remained positive *ie*. superior to 1 and the mesenchymal phenotype of cells was clearly maintained in OptiPASS medium.

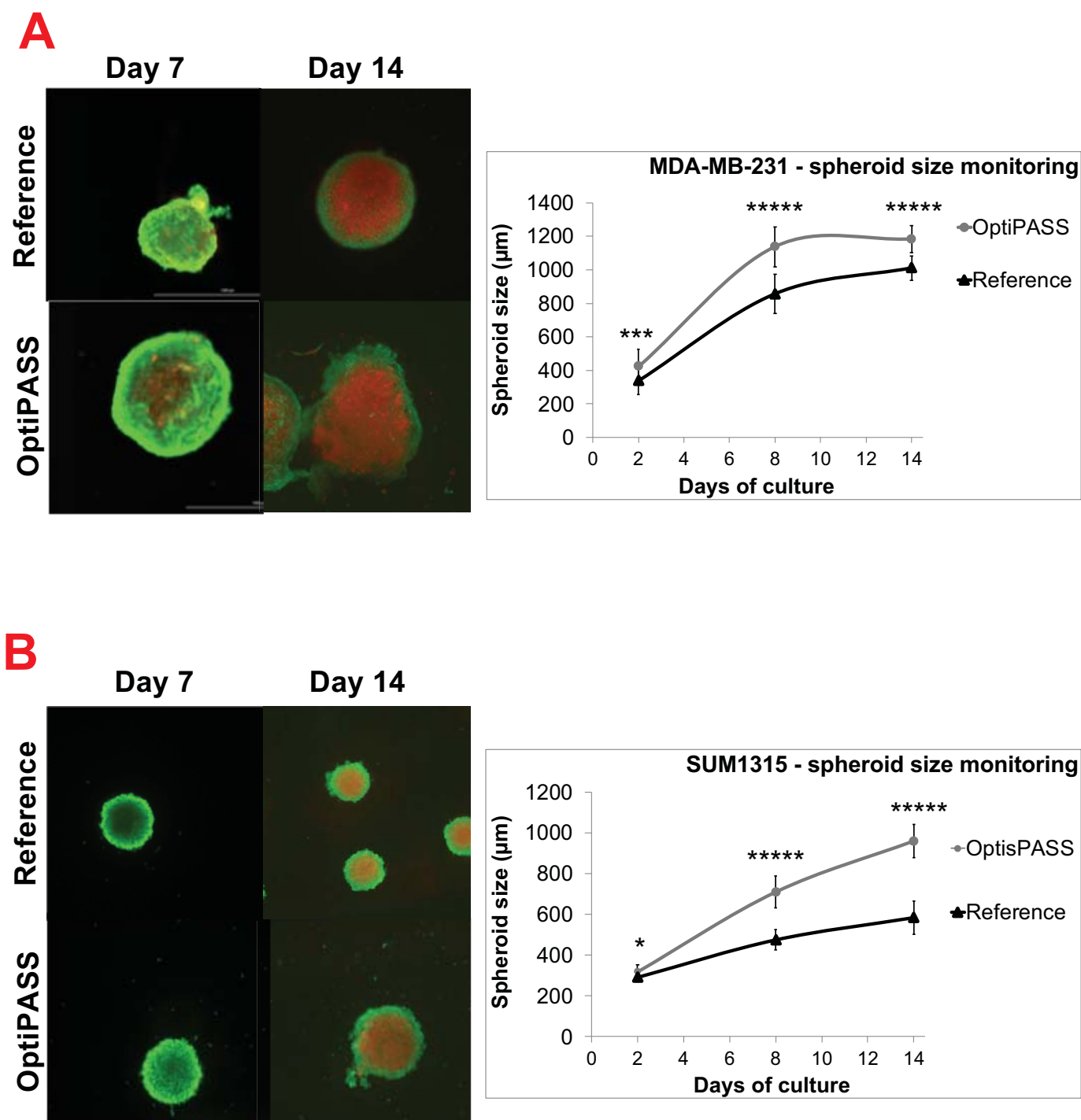


Fig 2. MDA-MB-231 (A) and SUM1315 (B) spheroid development with OptiPASS medium. a: Spheroid size monitoring: spheroids formed with reference and OptiPASS medium were imaged after 2, 8 and 14 days of culture with Cytation3MV (BIOTEK). The mean size of each spheroid condition ($n=48$) was calculated by the “cellular analysis” algorithm of Gen5.03 software (BIOTEK), bars=s.e.m. **b: Spheroids viability/mortality profile:** after 7 and 14 days of culture, spheroids ($n=5$) were harvested and incubated with Live/Dead fluorescent probes. Green markings correspond to Calcein-AM penetration in viable cells, and red markings correspond to ethidium homodimer-1 penetration in dead/necrotic cells. Observations were made with Cytation3MV (GFP and Texas Red filters, objective = 10X). * = $p<0.05$, *** = $p<0.001$, ***** = $p<0.00001$, two-sided student’s t-test comparison.

2. Three-dimensional (3D) cell culture of TNBC cell lines with OptiPASS medium

2.1. Spheroid development in OptiPASS medium

OptiPASS medium performances were then compared to reference medium for the development of 3D cell cultures with MDA-MB-231 and SUM1315 Triple-Negative breast cancer cell lines. For these experiments, cell lines previously cultured in monolayer with reference or OptiPASS medium were harvested and cultured in 3D condition according to *Dubois et al.* (2017) with their respective destination medium. In these cell culture conditions, spheroid size monitoring was analyzed for 14 days of culture with Cytation3MV plate reader (BIOTEK). Parallely, spheroid viability was assessed with Live/Dead fluorescent markings at day 7 and day 14.

For MDA-MB-231 cell line, the mean spheroid sizes were of $339 \pm 85 \mu\text{m}$ and $422 \pm 102 \mu\text{m}$ at day 2 ($p=0.0002$) and increased to $856 \pm 117 \mu\text{m}$ and $1137 \pm 120 \mu\text{m}$ ($p=10^{-17}$) at day 8 for reference and OptiPASS medium, respectively (Fig 2Aa). Then, spheroid diameter reached a growth plateau at day 14 with $1011 \pm 73 \mu\text{m}$ and $1183 \pm 80 \mu\text{m}$ at day 14 ($p=10^{-13}$), for reference and OptiPASS medium, respectively (Fig 2Aa). With this cell line, the mean spheroid sizes with OptiPASS medium always remained superior to reference medium. Then, spheroid viability was assessed with Live/Dead kit (Molecular probes), marking viable cells in green (Calcein-AM penetration) and dead cells in red (ethidium homodimer-1 penetration). For both cell culture conditions at day 7, spheroids exhibited a majority of green markings (Fig 2aB). Then, at day 14, a core of dead cells (red markings), surrounded with a proliferative cell ring (green markings of viable cells) was detected. Parallely, spheroid topology and level of compaction remained constant throughout the experiment, whatever the cell culture condition (Fig2Ab). These results demonstrated same spheroid viability/mortality profiles between reference medium and OptiPASS medium, for MDA-MB-231 cell line.

Similarly, for SUM1315 cell line, the mean spheroid sizes at day 2 were of $292 \pm 21 \mu\text{m}$ and $319 \pm 34 \mu\text{m}$ ($p=0.02$) and increased to $476 \pm 51 \mu\text{m}$ and $711 \pm 77 \mu\text{m}$ at day 8 ($p=10^{-35}$) and to $585 \pm 82 \mu\text{m}$ and $961 \pm 81 \mu\text{m}$ at day 14 ($p=10^{-44}$) in reference and OptiPASS medium, respectively (Fig2Ba). Then, for both cell culture conditions, Live/Dead fluorescent analysis showed similar spheroid green markings (viable cells) after 7 days of culture (Fig 2Bb). This profile then changed after 14 days, with the presence of a visible dead core in spheroids (red markings) surrounded with viable cells, for reference and OptiPASS medium conditions (Fig

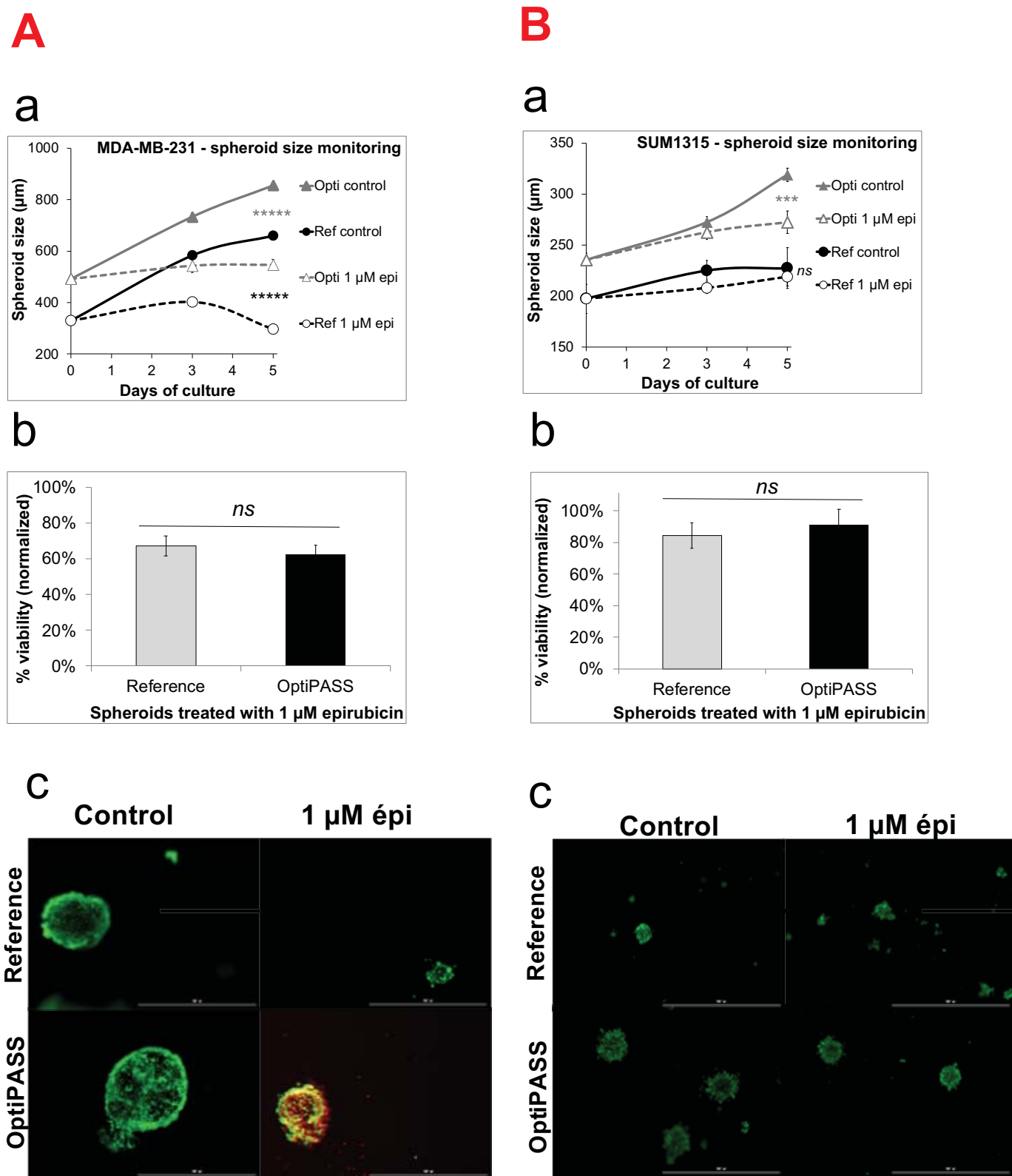


Fig 3. MDA-MB-231 (A) and SUM1315 (B) spheroids sensitivity to epirubicin in OptiPASS medium. Spheroids formed with reference or OptiPASS medium were treated with 1 µM of epirubicin for 5 days. **a: Spheroid size monitoring after epirubicin treatment:** the mean size of control and treated spheroids was measured for the 5 days of treatment with Cytation3MV (BIOTEK), $n=6$, bars=s.d.. **b: Spheroid viability after epirubicin treatment:** the percentage of treated spheroids viability was determined by the ratio of metabolic activity of treated spheroids on untreated spheroids at day 5. Then, the viability was normalized on the percentage of treated spheroid size (on control spheroid size) calculated at day 5 from each cell culture medium, $n=6$, bars=s.e.m. **c: Spheroid viability/mortality profile after epirubicin treatment:** after 5 days of treatment, control and treated spheroids were incubated with Calcein-AM and ethidium-homodimer-1 (Live/dead kit). green = viable cells, red = dead cells, $n=5$. Scale bar = 1000 µm. *ns* = non-significantly different, *** = $p<0.001$, two-sided student's t-test comparison.

2Bb). Furthermore, same spheroid compaction and topology was found in SUM1315 spheroids cultured in OptiPASS medium compared to reference medium. As previously, spheroids formed with OptiPASS medium clearly presented sizes superior to reference medium conditions. More, similar viability/mortality profiles were also detected between spheroids cultured in OptiPASS compared to reference.

2.2. Spheroid sensitivity to drugs in OptiPASS medium

Then, spheroid sensitivity to the anticancer agent epirubicin was studied in OptiPASS medium in comparison to reference medium. For this, spheroids of MDA-MB-231 and SUM1315 cell lines were treated with 1 μ M epirubicin for 5 days. First, the evolution of spheroid size was monitored for the 5 days of treatment (Fig 3Aa). Then, resazurin viability test (Fig 3Ab) and Live/Dead fluorescent analysis (Fig 3Ac) were performed at the end of the treatment on the 1 μ M epirubicin-treated spheroids in comparison with control spheroids.

In reference medium, MDA-MB-231 spheroid size increased from 330 ± 16 μ m (at day 0) to 659 ± 11 μ m (at day 5, $p=10^{-12}$). After treatment with 1 μ M epirubicin, it increased slightly to 402 ± 14 μ m after 3 days of treatment ($p=0.004$, compared to day 0), and then decreased significantly to 297 ± 62 μ m ($p=0.0002$, compared to day 3). With OptiPASS medium, control spheroid size increased significantly from 491 ± 8 μ m at day 0 to 855 ± 53 μ m at day 5 ($p=10^{-34}$). In contrast, epirubicin-treated spheroids size increased slightly to day 3 with 542 ± 25 μ m ($p=0.04$, compared to day 0) and remained stable until day 5 ($p=0.84$). Thereby, for the 5 days of 1 μ M epirubicin treatment, spheroids from both cell culture medium conditions showed significantly decreased sizes compared to their respective untreated controls. This highlights the cytostatic activity of epirubicin on these both cell culture media. Then, the percentage of spheroid viability (normalized to spheroid size) was determined after five days of treatment with epirubicin (Fig 3Ab), by the ratio of treated spheroids metabolic activity on untreated spheroids activity. For MDA-MB-231 cell line, the percentages of treated spheroids viability were similar between the two culture medium conditions, with $67 \pm 5\%$ for reference spheroids and $62 \pm 6\%$ for OptiPASS spheroids ($p=0.15$) (Fig 3Ab). Finally, live/dead markings were processed on both control and treated spheroids after five days of treatment (Fig 3Ac). The results showed that epirubicin-treated spheroids cultured in reference medium presented overall green markings, demonstrating the presence of mainly viable cells composing the spheroids, similarly as controls. In contrast, the analysis of treated spheroids cultured in OptiPASS medium showed clearly the presence of dead cells (red markings) compared to controls, revealing also a higher cytotoxic response with this chemotherapeutic agent.

Parallely, SUM1315 spheroids sensitivity to epirubicin was determined in OptiPASS medium and compared to reference medium. Control SUM1315 spheroids size did not increase in reference medium for the 5 days of treatment with $197 \pm 14 \mu\text{m}$ at day 0 and $227 \pm 8 \mu\text{m}$ at day 5 ($p=0.07$). Similarly, epirubicin-treated spheroids size remained stable for the 5 days of culture with $219 \pm 9 \mu\text{m}$ at day 5 ($p=0.21$ compared to day 0). More, no significant difference in spheroids size was detected between epirubicin treated spheroids and control, in reference medium ($p=0.13$). In contrast, with OptiPASS medium, SUM1315 spheroids size increased from $235 \pm 6 \mu\text{m}$ at day 0 to $319 \pm 6 \mu\text{m}$ at day 5 ($p=10^{-14}$). Parallely, epirubicin treated spheroids size increased until day 3 with $263 \pm 7 \mu\text{m}$ ($p=0.02$, compared to day 0), but remained stable until day 5 ($272 \pm 11 \mu\text{m}$, $p=0.91$ compared to day 3). Thus, with this cell line, a significant difference in spheroid size between untreated and treated conditions was detected at day 5, only when spheroids were cultured in OptiPASS medium ($p=0.0001$). This showed the cytostatic action of epirubicin on SUM1315 spheroids only with OptiPASS medium. Then, the analysis of spheroid viability after five days of treatment showed similar percentages of viability of $84 \pm 8\%$ and $91 \pm 10\%$ ($p=0.23$), for reference treated spheroids and OptiPASS treated spheroids, respectively. Finally, Live/dead markings showed similar viability patterns (green markings) between reference and OptiPASS culture conditions, and this for control and epirubicin-treated spheroids.

DISCUSSION

Preclinical studies aim to screen new drugs or new treatment strategies. These consist in several steps, including *in vitro* cell culture modelling. For this, the culture medium plays a crucial role on the metabolism and growth of cells in culture. As a result, the stability and homogeneity of culture medium components are essential factors for ensuring the proper cell response to drugs in *in vitro* screenings^{19–21}. Otherwise, culture media might need the adding of animal-serum in their composition, in order to ensure and maintain cell development and homeostasis. Nevertheless, the use of serum is controversial in the cell culture field, principally because of its heterogeneity. The challenge then appears to be the removal of serum from media, by using new synthetic formulations. In this context, the objective of this study was to determine the performances of a new synthetic serum-free medium OptiPASS (BIOPASS) adapted for the culture of mesenchymal stem cells, which formulation is an industrial secret. These works were processed in monolayer and 3D cell culture conditions, using these TNBC cell culture models cultured in OptiPASS medium or their serum-containing-reference media, RPMI1640 medium (supplemented with 10% FCS) and Ham's F12 medium (supplemented with 5% FCS), respectively.

Firstly, OptiPASS medium performances were determined in monolayer cell culture by the analysis of cell adherence, cell proliferation and cell metabolic activity. Then the expression of the mesenchymal phenotype marker vimentin was determined. Indeed, vimentin is the most commonly used marker for the determination of a phenotype change from epithelial to mesenchymal^{22,15,23,24}. Thus, for OptiPASS cell culture development in our experimental conditions, it was necessary to add a coating step before cell seeding. This latter was carried out with the deposition of 2% Geltrex solution containing extracellular matrix proteins, in order to trigger cell adherence²⁵. Thus, this Geltrex concentration was already determined and used for the development of 3D cell cultures for both MDA-MB-231 and SUM1315 cell lines¹⁸. With MDA-MB-231 cell line model, for all studied parameters, same cell adherence (78% vs 74%, $p=0.64$), cell proliferation rates (3.0 vs 3.4, $p=0.05$), cell metabolic activity (0.335 vs 0.366 at day 7, $p=0.40$) and same cellular carpet morphology were detected in RPMI1640 reference medium serum-supplemented and in OptiPASS medium, respectively. Similarly, the cellular expression of the mesenchymal marker vimentin in OptiPASS medium remained comparable to reference medium, showing the absence of cell line drift with this culture condition. These results suggest that OptiPASS medium is suitable for the monolayer culture of MDA-MB-231 cell line, providing same performances as the reference medium. In contrast, with SUM1315 cell line, lower (i) cell adherence (66% vs 22%, $p=0.01$), (ii) cell proliferation rates (3.3 vs 1.9, $p=10^{-12}$) and (iii) cell metabolic activity (0.148 vs 0.067, at day 7, $p=10^{-6}$) were detected in reference medium compared to OptiPASS medium, respectively. More, SUM1315 cells mostly presented an ovoid shape in bright field microscopy. These results suggest that cells cultured with OptiPASS medium may partly not adhere to the Geltrex coating or may detach. This may cause the decreased values of cell proliferation rates and metabolic activity for this cell line, as the cells may have detached prematurely. More, SUM1315 mesenchymal phenotype was still maintained in OptiPASS medium as depicted with the vimentin staining²⁶. Thus, the higher vimentin immunostaining intensity of SUM1315 floating-cells cultured in OptiPASS may be explained by their ovoid shape, in comparison with spread and attached cells culture in reference medium. Besides, in OptiPASS medium, the SUM1315 cell proportion profile revealed the presence of a majority of viable cells in the supernatant (74% for OptiPASS vs 25% for reference at day 7, $p=0.01$). All these results showed that OptiPASS medium was adapted for the expansion of MDA-MB-231 and SUM1315 cell lines cultured in monolayer, allowing to proceed to further 3D cell culture production steps.

Since few decades, new cell cultures models in three-dimensions (3D cell cultures) have been dramatically developing, as they reproduce more closely tumor's microenvironment^{27,28}, and are increasingly incremented into the drug development pipelines of preclinical studies^{13,29}. Thus, our team already developed 3D cell cultures on TNBC cell lines, highlighting

two different models *ie.* SUM1315 non-proliferative and MDA-MB-231 proliferative spheroids, in their respective serum-containing reference media ¹⁸. In this context, OptiPASS performances were tested on these 3D specific models. For this, spheroid size monitoring, spheroid viability/mortality monitoring and spheroid sensitivity to anticancer drugs were assessed on both cell lines cultured in OptiPASS and compared to reference medium. First, the analysis of spheroid size evolution showed a 1.3 and 1.5-fold increase in spheroid size when cultured with OptiPASS compared to reference medium, for MDA-MB-231 and SUM1315 cell lines, respectively. Then, the viability/mortality fluorescent experiments showed same profiles between OptiPASS and reference-produced spheroids. More, the formation of a necrotic core surrounded by a ring of viable/proliferating cells was clearly observed at day 14 for both culture conditions. This might be explained by the formation of nutrient and oxygen gradients within the spheroids, thus reproducing the *in vivo* behavior of tumours, as already described in literature ¹⁶. All of these results demonstrated that OptiPASS medium is very suitable for the 3D cell culture of TNBC cell lines MDA-MB-231 and SUM1315. More, it promoted enhanced spheroid proliferation rates, especially for SUM1315 cell line, turning this cell line into a high proliferative model in this culture condition.

Then, in order to use 3D cell cultures produced in OptiPASS medium for therapeutic drug screenings, spheroid sensitivity to epirubicin was determined in OptiPASS and compared to the reference medium. Spheroids were treated with epirubicin, a strong intercalating agent used in conventional chemotherapeutics protocols, presenting high cytostatic (slowed or stopped proliferation rate) and cytotoxic (decrease in viability and/or presence of dead cells) activities. For MDA-MB-231 spheroids treated with 1 μ M epirubicin in reference medium, a 2.2-fold decrease in size as well as a decrease in viability (67%) were detected compared to controls. Similarly, MDA-MB-231 spheroids cultured in OptiPASS medium presented a 1.6 fold size decrease after treatment associated with lowered viability (62%, similar to reference). Therefore, for this cell line, the cytostatic and cytotoxic actions of epirubicin were detected whatever the culture condition (reference or OptiPASS). In contrast, for SUM1315 spheroids cultured in reference medium, no difference in spheroid size was noted after the treatment, but only a viability drop (84% viability). Conversely, SUM1315 spheroids cultured in OptiPASS presented a 1.2-fold size spheroid decrease with slowed viability (91%, similar to reference). These results showed that the culture with OptiPASS medium allowed to detect the epirubicin cytostatic and cytotoxic actions on both cell lines.

All these results showed that proliferation, viability and drug sensibility of 3D cell cultures with MDA-MB-231 and SUM1315 cell lines were optimized in OptiPASS medium. This serum-free medium developed by BIOPASS is adapted to the culture of mesenchymal stem

cells and meets all quality control requirements for the culture of these specific cells. The experiments performed in this study showed that OptiPASS is suitable for the monolayer and the three-dimensional cell culture of these two triple negative breast cancer cell lines models. More, this method allowed a spheroid formation reproducibility per plate superior to 98%. These results also highlight the key role of the culture medium for the optimization of culture models' predictive response within the drug screening pipeline and open the way to the common use of this medium in preclinical breast cancer research studies. These research works aimed to modeling the development of two TNBC cell lines in a "standard and controlled" culture medium for optimizing *in vitro* predictive drug response. This methodology could be extended to other models of TNBC cell cultures or other cell lines. Other experiments aiming to determine OptiPASS performances with other cancer cell lines are currently underway.

METHODS

1. Maintenance of cell cultures

The triple-Negative Breast Cancer cell lines (TNBC) MDA-MB-231 (ATCC HTB-26) and SUM1315 (MO2, ASTERAND) were maintained in a humidified incubator at 37 °C under 5% CO₂. Cell culture was processed in 75 cm² culture flasks (FALCON) with 15 mL of the appropriate culture medium. For culture of MDA-MB-231 line, the reference medium was the RPMI 1640 medium (GIBCO) supplemented with fetal calf serum (FCS, 10%, EUROBIO) and gentamycin (20 µg/mL, PANPHARMA). For the SUM1315 cell line, the reference medium was the Ham's F-12 medium (GIBCO), supplemented with 5% FCS, 10 mM HEPES buffer (SIGMA), insulin (4 µg/mL, NOVO NORDISK A/S) and Epidermal Growth Factor (EGF, 10 ng/mL, SIGMA). These two respective media were untitled "reference" in experiments with both cell lines. For experiments with OptiPASS medium (BIOPASS), the latter was supplemented with gentamycin (20 µg/mL) and was untitled "OptiPASS" in our experiments. Cells were only cultured before passage number 30 to prevent cell line drift.

2. 2D cell culture development

2.1. Cell seeding

For both cell lines, cells were seeded at least in triplicates for each culture condition, at concentrations of 2×10^4 cells per cm^2 in 24-well microplates or 6-well microplates (FALCON) in 2 or 3 mL of culture medium, respectively. For cultures with OptiPASS medium, prior to seeding, wells were coated for 1h hour under agitation with 2% Geltrex diluted in medium.

2.2. Cell counting with Cytation3MV plate reader and trypan blue

The cellular carpet of each microplate wells was imaged daily with the Cytation3MV plate reader (BIOTEK, M=4X) coupled to Gen 5.03 software, until cells have reached the confluence (day 7). The number of living-adherent cells was then calculated for each culture condition by the “*cellular analysis*” algorithm of Gen 5.03 software, with the following settings: *threshold value = 1200, background = light, min object size = 15 μm , max object size = 50 μm* . The proliferation ratio was calculated by dividing the cell number of each well at day X by the number of cells one day after seeding (day 1).

Then, when cells were at confluence, the supernatant containing floating cells (living and dead) was collected. Adherent cells were trypsinized and also collected, allowing the determination of “adherent”, “floating” and “dead” cells proportion of each cell culture condition, by a trypan blue exclusion test.

2.3. Metabolic activity monitoring with resazurin test

Cell metabolic activity was determined by the resazurin test after 1, 3, 5 and 7 days of culture (confluence) in reference or OptiPASS medium. It is a colorimetric assay involving the reduction of resazurin (non-fluorescent) into resorufin (fluorescent, *ex/em* 570/584 nm) by the metabolically active cells. For this, the cellular carpet of each condition was rinsed in hot D-PBS and incubated with 100 μL of 60 μM resazurin (ACROS ORGANICS). The quantity of resorufin formed was monitored with Cytation3MV plate reader and presented in the graphs as the metabolic activity of cells $\pm$ s.d (standard deviation).

2.4. Expression of the mesenchymal marker vimentin by immunofluorescence

SUM1315 and MDA-MB-231 cells cultured with reference or OptiPASS medium were seeded in Ibitreat microplates (IBIDI) at concentrations of 5×10^5 cells per well. For cells cultured with OptiPASS medium, a 1h-coating with 2% Geltrex (in medium) was realized prior seeding. 24h after seeding, cells were fixed for 20 min with a solution of 4% paraformaldehyde (SIGMA) and permeabilized in ethanol 70° at -20°C for 5 min. Cells were incubated for 1 hour with a saturation solution (0.1/1% v/w Triton/BSA in PBS) and then for 1 hour with the primary

antibody (Vimentin, Mouse, Isotype IgG1a, 1/250 in PBS 0.1% triton) or the isotype control (1/1000, isotype IgG1a mouse, THERMOFISHER). Then, cells were incubated with the anti-mouse secondary antibody (1/400, AlexaFluor 647, MOLECULAR PROBES) for 1 hour, and then with 10 µg/mL Hoechst 33258 (SIGMA) for nuclear counterstaining. Finally, cells were dried, mounted with ProlongDiamond (THERMOFISHER) and observed with Cytation3MV plate reader (Dapi and Cy5 cube fluorescent filter cubes).

3. 3D cell culture development

3.1. Breast cancer spheroids production by the “liquid overlay” 3D technique

3D cell culture protocol with TNBC cell lines was processed according to *Dubois et. al*¹⁸. Briefly, cells cultured in reference or OptiPASS medium, respectively, were seeded in 96-well “Ultra-Low-Attachment” microplates (CORNING, 4520) at a concentration of 1000 cells per well and left in the incubator to let the cells aggregate in the bottom of the wells. After 24h of incubation, a cold solution of Geltrex LDEV-free (GIBCO, A14132-02) diluted in the appropriate culture medium (reference or OptiPASS) was dispensed in each well to a final concentration of 2%. Microplates were then agitated for 20 min at 185 rpm and left in the incubator for 24 hours. After this incubation time, unique compact spheroids were formed with reproducibility superior to 98% for both culture conditions.

3.2. Characterization of spheroid size and morphology with Cytation3MV

Spheroids formed with the two culture conditions were imaged daily (M=4X) from day 1 (day of effective spheroid formation) to day 14, with Cytation3MV coupled to Gen 5.03 software. The size of each spheroid was measured with the “*cellular analysis*” algorithm of the software with the following settings: *threshold value = 10000, background = light, min object size = 180 µm, max object size = 1000 µm*.

3.3. Spheroid viability monitoring with Live/Dead fluorescent probes

Spheroid viability was monitored after seven and fourteen days of culture (day 7, day 14) by the Live/Dead Kit (MOLECULAR PROBES, L3224), marking viable cells in green (calcein-AM penetration) and dead cells in red (ethidium homodimer-1 penetration). For this, spheroids were harvested and deposited into 6-well microplates. Spheroids were rinsed twice with hot D-PBS and incubated with a solution of 2 µM calcein-AM and 4 µM etdh-1 in D-PBS

for 45 min (protected from light), according to supplier's instructions. Spheroids were then transferred into 8-well uncoated micro slides (IBIDI, 80821) and imaged on Cytation3MV plate reader with green fluorescent protein cube (GFP) and Propidium Iodide (PI) fluorescence cube loaded on the device (M=4X).

3.4. Spheroid anticancer drug sensitivity determination

Spheroids formed in reference medium or OptiPASS medium were treated with 1 μ M of epirubicin (stock solution of 1 mM dissolved in distilled water), for 5 days. The size of control and 1 μ M-treated spheroids was monitored with Cytation3MV for 5 days, as previously described. The percentage of treated spheroids cell viability at day 5 was determined with resazurin test, by the ratio of resorufin formed in treated spheroids on control spheroids. These values were then normalized on the percentage of spheroids treated size on control size, at day 5. Finally, the viability assay Live/Dead was performed on treated and control spheroids at day 5.

4. Statistical analysis

Results were expressed as means $\pm$ standard deviation (s.d.) or standard error (s.e.m) of n independent experiments. Every experiment was performed at least in triplicate and then statistically compared using a two-sided student's t-test. Data comparison was considered statistically significant when the p value was $p < 0.05$ (*). Lower p values were noted in figures as follows: $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****) and $p < 0.00001$ (*****). Non-significant results were noted as "ns".

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AUTHOR CONTRIBUTIONS

C.D., P.D., C.A., J.G., P.V., B.V., E.M., F.P.L. and M.B. conceived and designed the study; C.D. performed the experimental work; C.D., P.D., B.V., J.G., E.M. and M.B. analyzed the data; C.D. performed statistical analysis; C.D. and M.B. wrote the manuscript. All authors reviewed the manuscript.

ADDITIONAL INFORMATION

Competing interests: The authors declare no competing financial interests.

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

III) Conclusion sur les travaux

Ces travaux ont permis de développer dans un nouveau milieu synthétique et contrôlé (sans sérum de veau fœtal ajouté) intitulé « **OptiPASS** », la culture de deux lignées cellulaires MDA-MB-231 et SUM1315 de cancer du sein TN, en conditions 2D et 3D. Pour chaque lignée cellulaire, l'ensemble des analyses ont été comparées à celles du milieu de référence, recommandé par le fournisseur. Pour la lignée MDA-MB-231, l'adhérence, la prolifération et le phénotype cellulaire ont été conservés pour les deux conditions 2D et 3D dans le milieu **OptiPASS**, en comparaison avec leur milieu de référence. De plus, les sphéroïdes formés dans le milieu **OptiPASS** présentent une plus forte prolifération, permettant ainsi de mettre en évidence l'activité cytostatique et cytotoxique de l'épirubicine, l'anticancéreux testé. Par contre, pour la lignée SUM1315, la prolifération des cultures cellulaires en monocouche en présence du milieu **OptiPASS** s'est avérée diminuée en comparaison du milieu de référence. Ceci peut être expliqué par une plus faible adhérence des cellules aux protéines matricielles utilisées pour le *coating* des supports. Cependant, la prolifération cellulaire en condition 2D reste suffisante afin de les utiliser pour la formation des sphéroïdes. De plus, les sphéroïdes SUM1315 formés dans du milieu **OptiPASS** présentent une forte capacité de prolifération, ce qui n'est le cas dans leur milieu de référence. Cette capacité proliférative a permis de mettre en évidence sur cette lignée TN, l'efficacité cytostatique de l'épirubicine en plus de son activité cytotoxique. De plus, le maintien de l'expression du marqueur mésenchymateux, la vimentine, dans les deux lignées a permis de confirmer l'absence de dérive de la lignée cultivée dans ce milieu **OptiPASS**.

L'ensemble de ces travaux ont permis de valider l'utilisation du milieu **OptiPASS** pour la culture des lignées de cancer du sein TN en trois dimensions. Ces résultats sont encourageants et ouvrent de nouvelles perspectives quant à l'utilisation de ce milieu synthétique dans les tests de screening et de caractérisation des molécules chimiothérapeutiques en développement. En effet, la démocratisation d'un milieu de culture reproductible et fiable permettrait d'uniformiser les résultats biologiques, et limiterait l'usage d'éléments d'origine animale. Dans cette optique, le milieu **OptiPASS** est actuellement en cours de développement sur d'autres modèles cellulaires tumoraux et sains.

C) Optimisation du traitement du cancer du sein triple-négatif : modélisation d'un traitement associant la thérapie ciblée anti-PARP1 Olaparib® et la radiothérapie fractionnée sur des cultures cellulaires en trois dimensions des lignées TNBC SUM1315 et MDA-MB-231.

I) Introduction

Les inhibiteurs de PARP1 ouvrent de nouvelles perspectives d'amélioration du traitement des cancers du sein « Triple-Négatifs ». En effet, ils constituent une nouvelle classe de thérapeutiques ciblant les déficiences dans les systèmes de réparation de l'ADN propres aux tumeurs mammaires TN, *via* le concept de létalité synthétique. Afin d'augmenter l'efficacité des traitements anti-PARPs, des stratégies de co-traitement sont évaluées dans les études précliniques et cliniques. A titre d'exemple, des traitements combinant les anti-PARPs avec des agents induisant des cassures de l'ADN (**Duma et al. 2018**) tels que des sels de platine, des agents alkylants, des inhibiteurs de topoisomérases, mais également combinant des traitements par immunothérapie ou la radiothérapie, sont actuellement en cours d'étude (**Xin Wang et al. 2018; O'Sullivan et al. 2014; Lesueur et al. 2017**). Cette dernière stratégie présente un rationnel fort de par le double ciblage spécifique de ces deux traitements (Olaparib + Radiothérapie) (**Pernin et al. 2014**). En effet, les inhibiteurs de PARP1 induisent une augmentation du taux des cassures simple-brin qui dégénèrent en cassures double-brin s'accumulant dans le noyau des cellules déficientes pour le système HR. Cette action, conduit alors à une accumulation des erreurs induisant la mortalité spécifique des cellules tumorales. De même, la cytotoxicité de l'irradiation est principalement liée au taux de cassures de l'ADN double brin, engendrant une accumulation de lésions létales et sublétales (**Chalmers et al. 2010**). Par conséquent, l'inhibition de PARP1 augmenterait la radiosensibilité des cellules tumorales.

Dans ce contexte, la deuxième partie des travaux de recherche de ma thèse a porté sur l'optimisation du traitement des tumeurs mammaires triple-négatives par l'étude de

l'efficacité d'un traitement combinant l'Olaparib (anti-PARP1) et la radiothérapie fractionnée (5x2 Gy). Les études de toxicité ont été réalisées sur des modèles de culture cellulaires en 2D, par l'analyse de l'induction des cassures double-brin, de la survie clonogénique* et de la survie cellulaire globale. Par la suite, la toxicité à long terme (6, 8 et 10 jours) du co-traitement a été déterminée sur les modèles de culture en 3D, par l'analyse de la taille et de la viabilité des sphéroïdes.

Ces travaux de recherches ont été réalisés en collaboration avec les services de Physique Médicale et de Radiothérapie du Centre de Lutte Contre le Cancer Jean Perrin et **font l'objet d'une publication en premier auteur.**

II) (Article rédigé) Optimization of Triple-Negative breast cancer treatment: Modelization of a cotreatment combining the anti-PARP1 targeted therapy Olaparib® and fractioned radiotherapy on three-dimensional SUM1315 and MDA-MB-231 cell cultures.

Optimization of Triple-Negative breast cancer treatment: Modelization of a cotreatment combining the anti-PARP1 targeted therapy Olaparib® and fractioned radiotherapy on two and three-dimensional MDA-MB-231 and SUM1315 cell cultures.

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ABSTRACT

Breast cancer is a very complex and heterogeneous disease. Among the multiple molecular subtypes, the “Triple-Negative Breast Cancer” subtype (TNBC) is particularly aggressive. Indeed, the absence of hormone receptors expression *ie.* progesterone and estrogen as well as the absence of HER2 overexpression in this tumours lead to limited treatment strategies and a poor prognosis. To improve the patient outcome, targeted therapies such as Poly-ADP-ribose Polymerase inhibitors - PARPi or anti-PARPs - were developed in preclinical and clinical studies, such as Olaparib. In addition to being used as monotherapy treatment agents, PARP1 inhibitors, aiming to increase the number of lethal DNA double-stranded break in tumour cells, may be used in combination with radiotherapy. Thus, the effectiveness of a combinatory treatment combining the anti-PARP Olaparib and fractioned radiotherapy was studied on two TNBC models - MDA-MB-231 and SUM1315 cell lines - cultured in 2D and 3D conditions. Indeed, 3D cell cultures are increasingly developed and used in preclinical studies as these models mimic more precisely the tumour microenvironment and the metabolic and cellular gradients. These experiments showed the interest of the combinatory regimen including Olaparib and fractioned radiotherapy - 2 gy / fraction - on the decrease in clonogenic viability, overall viability in 2D, and on the long-term growth inhibition and toxicity in spheroids cultured from Triple-Negative breast cancer cell lines. More, these works led to the promising perspective of a (i) daily (ii) low dose and (iii) long-term Olaparib administration coupled to fractioned radiotherapy on resistant metastatic breast cancers, and possibly for brain localizations, given the very difficult handling of these advanced stages.

KEYWORDS

Triple-Negative breast cancer, TNBC, anti-PARP Olaparib, fractioned radiotherapy, co-treatment, 3D cell cultures, spheroid, transcriptomic analysis

INTRODUCTION

Breast cancer is a very complex disease, presenting various phenotypes with distinct prognoses and responses to treatment (**Horton et al., 2018**). Among the multiple classifications, the “Triple-Negative Breast Cancer” subtype (TNBC) represents 12 to 17% of breast cancers (**Foulkes et al., 2010; Badve et al., 2011**) and is sub-divided into several transcriptomic molecular sub-classes such as basal-like 1 and 2 (BL1/2), mesenchymal (M) and luminal androgen receptor (LAR) (**Lehmann and Pietenpol, 2014; Lehmann et al., 2016**). TNBC tumours are mainly characterized by the absence of estrogen and progesterone receptor expression, the absence of Human-Epidermal-Receptor Type 2 overexpression, a high positivity for the Ki67 proliferation marker, and the expression of the cytokeratins 5 and 6 (Penault-Llorca and Viale, 2012). More, a great proportion of TNBC tumours carry hereditary mutations into the BReast CAncer early onset 1 and 2 genes (BRCA1/2 genes) (**Kobayashi, 2008; Rakha et al., 2009; de Ruijter et al., 2011**), which are responsible for hereditary predisposed cancers developments.

In clinics, a great proportion of TNBC tumours is not sensitive to conventional anticancer treatments such as chemotherapy or radiotherapy, involving a high number of relapses and a high mortality. Despite this very aggressive profile, there is currently no targeted treatment and/or specific management for this breast cancer subtype. As a result, the treatment is often composed of surgery followed by loco-regional radiotherapy and conventional chemotherapy. In this pathway, the adjuvant radiotherapy can destroy the micro-metastases still present in the surgical area, thus avoiding 70% of relapses and long-term mortality (**Vinh-Hung and Verschraegen, 2004**). Moreover, when the tumour size is very important, or in the case of an inflammatory breast cancer, radiotherapy can take place in the neo-adjuvant setting before surgery, in order to facilitate the surgical procedure. In this context, a complete pathological response can be obtained in particular in combination with chemotherapy (**Liedtke et al., 2008**). The latter, which is the only systemic treatment available, is the only therapy currently recommended in the metastatic phase for this subtype. However, these TNBC tumours temporarily respond to adjuvant therapies such as chemotherapy or radiotherapy, but then a rapid and almost systematic relapse associated with the development of metastases is detected in almost 60% of patients (**Foulkes et al., 2010**). As a result, many efforts have been made to improve the prognosis of patients, by the optimization of treatment protocols and the development of new-targeted therapeutic agents exploiting the specificities of TNBC tumours. The therapies in development such as Tyrosine Kinase Inhibitors (TKI), anti-Epidermal-Growth-Factor-Receptor (anti-EGFR) or Poly-ADP-Ribose-Polymerase 1 (PARP1) inhibitors,

open new perspectives to improve the treatment of these tumours (**Anders and Carey, 2009; Penault-Llorca and Viale, 2012**).

PARP1 inhibitors such as Olaparib® (AstraZeneca), constitute a new class of targeted therapeutics acting on DNA repair systems using the synthetic lethality concept. These open up new prospects for improving the treatment of this poor prognosis subtype of breast cancer (**Duma et al., 2018**). In addition to being used as monotherapy treatment agents, PARP1 inhibitors may be used in combination with chemotherapy or radiotherapy. These combination methods exhibit a strong rationality due to the inhibition of the DNA lesions mechanisms of repair induced by chemical agents and/or by irradiation (**Pernin et al., 2014**). Indeed, PARP1 inhibitors induce an increase in the rate of unrepaired DNA single-strand breaks in proliferating cells, leading to the more frequent appearance of lethal DNA double-stranded breaks. Similarly, the cytotoxicity of irradiation is mainly related to the rate of DNA double-strand breaks, due to the accumulation of lethal lesions and sub lethal lesions (**Chalmers et al., 2010**).

The pipeline of drug development is composed of several preclinical and clinical phases. The preclinical setting is divided into *in vitro* and *in vivo* experiments which aims are the determination of drug efficacy and toxicity. Recently, the three-dimensional cell culture has been integrated into these preclinical processes after the monolayer *in vitro* cell culture experiments. Indeed, the 3D cell culture appears to be an adapted tool for the evaluation of drug efficacy as it mimics more precisely the tumour microenvironment by the formation of genetical, biochemical and phenotypical gradients, resembling intratumoral gradients and microenvironment (**Costa et al., 2016; Dubois et al., 2017; Fang and Eglen, 2017**).

In this context, these works aimed to model the effectiveness of the co-treatment combining increasing concentrations of Olaparib and fractioned radiotherapy (5 x 2 Gy) on two TNBC models *i.e.* MDA-MB-231 and SUM1315 cultured in 2D and 3D conditions. The efficacy of this combinatory strategy was carried out by the determination of (i) DNA double strand breaks generation, (ii) cell survival and (iii) clonogenic potential in monolayer cell cultures. In parallel, the long-term effect of the co-treatment was assessed in more predictive models that are 3D cell cultures, by the analysis of spheroid growth inhibition and mortality/viability profiles. More, in order to define the difference in cell line sensitivity, a *in silico* transcriptomic approach was realized.

MATERIAL AND METHODS

1 Cell lines maintenance

The two Triple-Negative breast cancer cell lines, SUM1315 (Asterand®, MO2) and MDA-MB-231 (HTB-26™, ATCC®), were used in this study. SUM1315 cell line was cultured in Ham's F12 medium (Gibco®) supplemented with 5% decompemented fetal calf serum (Eurobio®), 10 mM HEPES buffer (Sigma®), 20 µg/mL gentamycin (Panpharma®), 10 ng/mL EGF (Sigma®) and 4 µg/mL insulin (NovoRapid®). For MDA-MB-231 cell line culture achievement, the RPMI medium 1640 (GIBCO®) was supplemented with 10% decompemented fetal calf serum (Eurobio®) and 20 µg/mL of gentamycin (Panpharma®). Both cell lines were maintained at 37°C under 5% CO₂ in a humidified incubator (Sanyo®). For both cell lines, cells were seeded in 15 ml of culture medium at a concentration of 5000 cells/cm² in 75 cm² Falcon® culture flasks, following supplier's instructions (**Dufour et al., 2015**). Before confluence, cells were trypsinized, counted with the blue trypan exclusion test and re-seeded into new culture flasks at the same cell concentration, or processed in the experiments.

2 Olaparib® solubilization

Olaparib (Carbosynth®) was solubilized in Dimethylsulfoxide (DMSO) at a concentration of 100 mM, constituting the stock solution. For all experiments, the solubilized Olaparib was diluted in the culture medium at different concentrations.

3 Cell culture irradiation

3.1 Device and technique of irradiation and dose calculation

The cells were irradiated on a linear electron accelerator (Truebeam®, Varian Medical Systems, Palo Alto) with a 6 MegaVolts X-ray beam. The cells were placed 3 cm deep inside a 13 cm thick PolyMethyl MethAcrylate (PMMA) phantom in order to obtain a homogeneous distribution of dose and respect the charged particle equilibrium conditions. A computed tomography (CT) scanner examination of this phantom was performed with each device containing the irradiated cells (culture flasks, 96-well flat-bottom microplates, 6-well plates and 96-well "Ultra-Low-Attachment" microplates). Examinations were carried out with the multi-slice CT scanner of the radiotherapy department (Lightspeed RT 16, General Electric

Healthcare). The acquired images were sent on the treatment planning system Eclipse version 13.5 (Varian Medical Systems, Palo Alto) in order to calculate the dose to be delivered to the cells. The dose was prescribed in the middle of the volume containing the cells and the calculation was performed with the Anisotropic Analytical Algorithm (AAA). This dose was delivered through a 25 cm * 25 cm beam whose axis projects perpendicular to the phantom surface and with a source - phantom surface distance of 100 cm. The dose rate applied to the accelerator was 400 Monitor Units per minute (MU/min), which corresponds to the dose rate conventionally used in radiotherapy.

3.2 Irradiation protocol

For the co-treatment experiments using both monolayer and three-dimensional cell cultures, Olaparib was added once three hours before the first radiotherapy session. The radiotherapy protocol was then carried out at a daily dose of 2 Gy for 1, 3 or 5 days, to obtain a final dose of 2, 6 or 10 Gy. The non-irradiated culture plates without Olaparib or treated with Olaparib® alone or with DMSO 0.1% were maintained in the incubator at 37°C under 5% CO₂ throughout all the experiment. The irradiated plates also remained in the same incubator outside of radiotherapy treatment sessions.

4 Monolayer cell culture experiments

4.1 Gamma H2AX Immunofluorescent markings

4.1.1 Protocol

For FOCI γ H2AX immunomarkings, cells were seeded into Ibittreat cell-culture-treated microscopy chambers (IBIDI®) at a concentration of 5000 cells/cm² in 300 μ L of medium and let in the incubator for 24 hours before the first treatments in order to allow their attachment to the support. Then, cells were treated with 5 and 50 μ M Olaparib for 3 hours and a single X-ray dose of 2 Gy was applied. One hour after the radiotherapy session, MDA-MB-231 and SUM1315 cells were fixed for 20 min with a solution of 4% paraformaldehyde in PBS (PAF, Sigma®) and permeabilized for 5 min in ethanol 70° at -20°C. Cells were then incubated for 1 hour with a saturation solution (0.1/1% Triton/BSA in PBS) and then for 1 hour with the primary antibody (Anti-phospho-histone H2AX, Ser 139 clone JBW301, Millipore) or the isotype control (1/1000, isotype IgG1a mouse, ThermoFisher®) diluted in PBS 0.1/0.1% Triton/BSA. Then, cells were incubated with the anti-mouse secondary antibody (1/800, Alexa Fluor 488 goat

anti-mouse IgG, Molecular probes®) for 1 hour, and then for 10 min with 10 µg/mL of Hoechst 33258 (Sigma®) for nuclear counterstaining. Finally, cells were dried, mounted with ProlongDiamond (Thermofisher®) and observed with CytationTM3MV plate reader (Biotek®) with GFP and DAPI filter cubes loaded in the device. All image sets were realised with the same camera settings (exposition time, LED intensity and camera gain).

4.1.2 FOCI γ H2AX quantification

The number of γ H2AX FOCI expressed in each nucleus was determined with ImageJ® software. First, the hoechst counterstaining mask of all cell nuclei allowed the isolation of every nucleus element of the images. Then, the number of γ H2AX FOCI in every nucleus was calculated with the “saturated pixel” method. For this, the number of all foci per image was enumerated and divided by the number of nuclei of the same image. This analysis was processed for each culture condition on > 10 fields, representing at least more than fifty cells. The results were represented as the mean number of γ H2AX foci per nucleus.

4.1.3 SulfoRhodamine B survival test

For cell survival experiments with Sulforhodamine B, cells were seeded into 96-well flat-bottom culture-treated microplates (Falcon® Microtest 96) at a concentration of 5000 cells per cm² in 100 µl of culture medium and let in the incubator for 24 hours before the first treatments in order to allow their attachment to the support. Cells were then treated with increasing concentrations of Olaparib of 0.1; 1; 5; 10; 25; 50; 75 and 100 µM for 24h, 72h or 120h, corresponding to concomitant doses of radiotherapy of 2, 6 and 10 Gy, respectively. SRB tests were performed 24 hours after each treatment endpoint. The SulfoRhodamine B binds to proteins, allowing the quantification of cell protein amount, and thus indirectly the quantity of cells. Briefly, 50 µL of a 50% (weight/volume) trichloroacetic acid (TCA) solution was added to each well for 1 hour at 4°C. Plates were then washed 3 times with water, dried, and 50 µL of a 0.4% (w/v) solution of SRB in 1% (v/v) acetic acid was added to each well. After 15 minutes at room temperature, plates were washed 3 times with 1% acetic acid solution and dried. 200 µl of a 10 mM Tris-base buffer solution was finally added to the wells and plates were stirred for 30 min. The optical density (OD) of SRB dye dissolved in each well was determined at 540 nm using a microplate reader (VWR Multiskan FC®). The absence of DMSO 0.1 toxicity was systematically checked prior to results analysis. Then, cell survival was calculated for each treatment condition by the comparison of 24h, 72h and 120h aged untreated control wells with the following formula:

$$\text{Cell survival (\%)} = [\text{OD}_{540\text{nm}}^{\text{treated wells}} / \text{OD}_{540\text{nm}}^{\text{no X-Ray/no Olaparib = control}}] \times 100.$$

The results are presented as mean viability of $n > 45$ wells $\pm$ their standard deviation (s.d.).

4.2 Clonogenic survival test

The clonogenic potential of cells after a treatment with radiotherapy alone or combined with Olaparib (0.5, 5 and 50 μM) was evaluated twenty-one hours after cumulative X-ray doses of 2, 6 or 10 Gy, corresponding to concomitant Olaparib expositions of 24h, 72h or 120h. For this, cells were seeded into 6-well plates (Falcon®) in 1.5 mL of culture medium at concentrations of 5000 and 2500 cells per cm^2 for SUM1315 and MDA-MB-231 cell lines, respectively and let in the incubator for 24 hours before the first treatments in order to allow their attachment to the support. Then, after each treatment endpoint, cells were recovered by trypsinisation and enumerated by a blue trypan exclusion test. Cells were then re-seeded into new 6-well plates, at a cell concentration adapted to each treatment condition (Olaparib® concentration and / or dose of radiotherapy). For each treatment condition, after a time corresponding to nine doubling times, the number of colonies formed was determined (objective 10X).

The "Plating Efficiency" (PE), corresponding to the cell repopulation factor after each treatment condition was calculated according to the following formula:

$$\text{PE} = \text{Number of colonies formed} / \text{Number of seeded cells at } T_0$$

Then, the survival fraction after each X-ray dose was calculated compared to the corresponding untreated control of each dose (= No X-ray and No Olaparib treatment) with the following formula:

$$\text{Survival fraction (\%)} = \frac{\text{PE treated condition (X-ray dose } \pm \text{ Olaparib)}}{\text{PE control (no X-ray/no Olaparib) of each corresponding X-ray dose}}$$

The values of the clonogenic survival were expressed as mean survival $\pm$ their standard deviation (s.d.) of $n = 5$ replicates.

5 Three-dimensional cell cultures experiments

5.1 Spheroid production by liquid overlay technique

For SUM1315 and MDA-MB-231 cell lines spheroids formation, the liquid overlay technique (LOT) was performed (**Dubois et al., 2017**). Briefly, cells were seeded into 96-well “Ultra-Low-Attachment” microplates (Corning®, 4520) at a concentration of 1000 cells/well in 100 µL of culture medium. 24h after cell seeding, a cold solution of Geltrex® was added to the culture medium for a final concentration of 2%. Plates were then shaken at 200 rpm for 20 min on a microplate agitator and then incubated for 24h until spheroid formation. Spheroid size follow-up was monitored with Cytation3®MV plate reader (Biotek®).

5.2 Spheroid treatment with Olaparib and fractioned radiotherapy

Spheroids were treated with 5 and 50 µM of Olaparib for 6, 8 and 10 days, corresponding to concomitant X-ray doses of 2 Gy (1 session), 6 Gy (3 successive daily sessions) and 10 Gy (5 successive daily sessions).

5.3 Spheroid size monitoring

The size of spheroids after each treatment endpoint (6, 8 and 10 days) was monitored with the Cytation™3MV microplate reader (Biotek®) using the “*cellular analysis*” algorithm of the Gen 5.03 software (Biotek®). Results were expressed as the mean spheroid size of each treatment condition (n > 45) with their standard error (s.d.).

5.4 Spheroid metabolic activity assessment with the resazurin test

The resazurin test is a colorimetric/fluorescent assay involving the reduction of resazurin (blue and non-fluorescent) in resorufin (pink and fluorescent, excitation/emission: 570/584 nm) by the mitochondria of metabolically active cells. In order to determine the metabolic activity of spheroids after a treatment with Olaparib alone or combined with fractioned radiotherapy, spheroids from every treatment condition were transferred one-by-one in a new 96-well microplate containing 60 µM resazurin in PBS. Then, the Fluorescence Intensity (FI) corresponding to the amount of resorufin formed was quantified in each well with Cytation3MV plate reader (quantitative fluorescence mode) after an optimal incubation time. This allowed the determination of the percentage of spheroid metabolic activity as controls calculated as follows:

$\text{Metabolic activity (\%)} = \frac{\text{FI treated spheroid (X-ray } \pm \text{ Olaparib or Olaparib } \pm \text{ X-ray)}}{\text{FI control spheroid (no Olaparib/no X-ray)}}$
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The results were presented as mean viability of each treatment condition (n > 16 spheroids) $\pm$ their standard deviation (s.d.).

5.5 Viability/Mortality fluorescent profile (Live/Dead)

The Live/dead fluorescent test allows the visualisation of living and dead cells forming the spheroids without fixation. For this, spheroids of each treatment condition (n=5) were harvested and rinsed twice in PBS. They were then incubated with 4 μ M ethidium-homodimer (Etdh-1, red fluorescence, penetrating dead cells) and 2 μ M Calcein-AM (green fluorescence, penetrating living cells) for 45 min. The fluorescence of each fluorophore was then imaged with Cytation3MV plate reader, with the GFP and Texas Red filter cubes loaded in the device for visualisation of Calcein-AM marking and Etdh-1 marking in spheroids, respectively. For the image analysis to be comparable, same exposure time, LED intensity and gain were programmed for all image sets.

6 Statistical analysis

For FOCl γ H2AX immunostaining (figure 1), clonogenic survival test (figure 2), spheroid size evolution and spheroid metabolic activity tests (figure 6), all collected and analyzed data were statistically compared with a Student's two-sided test, where significative values are indicated in the graphs as : *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 and *****p<0.00001.

Then, in order to describe the variation of cell survival (in 2D condition) or cell metabolic activity (in 3D condition) in the data set, the level of interaction between all treatment conditions (i.e. cell line type, Olaparib concentration, Olaparib time of exposition and radiotherapy dose) was determined with a multivariate ANOVA analysis (mixed model). This analysis was performed with the SEM software (developed by F.K., Centre Jean Perrin, Clermont-Fd) which compiled the results either obtained from (i) the SRB survival tests in monolayer cell culture (figure 4) or (ii) the spheroid metabolic activity determined by the resazurin test (figure 7).

7 Transcriptomic analysis of TNBC cell lines

All available MDA-MB-231 and SUM1315 transcriptomic data from different studies (**Boire et al., 2017; Daemen et al., 2013; Marcotte et al., 2016**) were collected from the NCBI public dataset GEO DataSet. Accession number, sample and sequencing information are indicated in the table below:

Name	Accession N°	Library type	Sequencing	End Type	Reference
MDAMB231_A	GSE73526	TruSeq Stranded mRNA	Illumina HiSeq 2000	paired-end	Marcotte R., Et al., 2016
MDAMB231_B	GSE48213	TruSeq RNA Sample Preparation Kit	Illumina Genome Analyzer IIx	paired-end	Daemen A., Et al., 2013
MDAMB231_C	GSE83132	TruSeq RNA Sample Prep Kit v2	Illumina HiSeq 2000	paired-end	Boire A., et al., 2017
MDAMB231_D	GSE83132	TruSeq RNA Sample Prep Kit v2	Illumina HiSeq 2000	paired-end	Boire A., et al., 2017
MDAMB231_E	GSE83132	TruSeq RNA Sample Prep Kit v2	Illumina HiSeq 2000	paired-end	Boire A., et al., 2017
SUM1315_B	GSE48213	TruSeq RNA Sample Preparation Kit	Illumina Genome Analyzer IIx	paired-end	Daemen A., Et al., 2013
SUM1315_A	GSE73526	TruSeq Stranded mRNA	Illumina HiSeq 2000	paired-end	Marcotte R., Et al., 2016

Fastq files were downloaded and analyzed on Genotoul Bio-informatic cluster. Input reads for each sample were aligned to the Human (GRCh38) reference genome using TopHat (**Trapnell et al., 2009**). Two-dimensional principal component analysis (PCA) of all data 500 most variant genes was performed to assess quality samples (Figure XX). It depicted that the first principal component represents 63% of variance and separated the two cell lines. The R package Deseq2 was used to analyse the statistically significant Differential Expressed Genes (DEG) between MDA-MB-231 and SUM1315 cell lines. An adjusted P value of <0.1 is the cutoff to select specifically DEG between the two cell lines. This DEGs list was subject as query to Ingenuity Pathway Analysis (IPA), (QIAGEN Inc., <https://www.qiagenbioinformatics.com/products/ingenuitypathway-analysis>). IPA permits to infer functional and cellular processes that are modified between these two cell lines using the Ingenuity Knowledge Base. Two statistical indexes (p-value and Z-score) are determined for each prediction. The p-value is calculated by a Fisher exact test and permits to identify significantly enriched pathways. The IPA z-score is the statistical measure of the match between altered genes and the inferred biological function. Z-score let to predicts the direction of change of a function. Indeed, a cellular function activity is increased when z-score is > 2 and decreased when z-score < - 2.

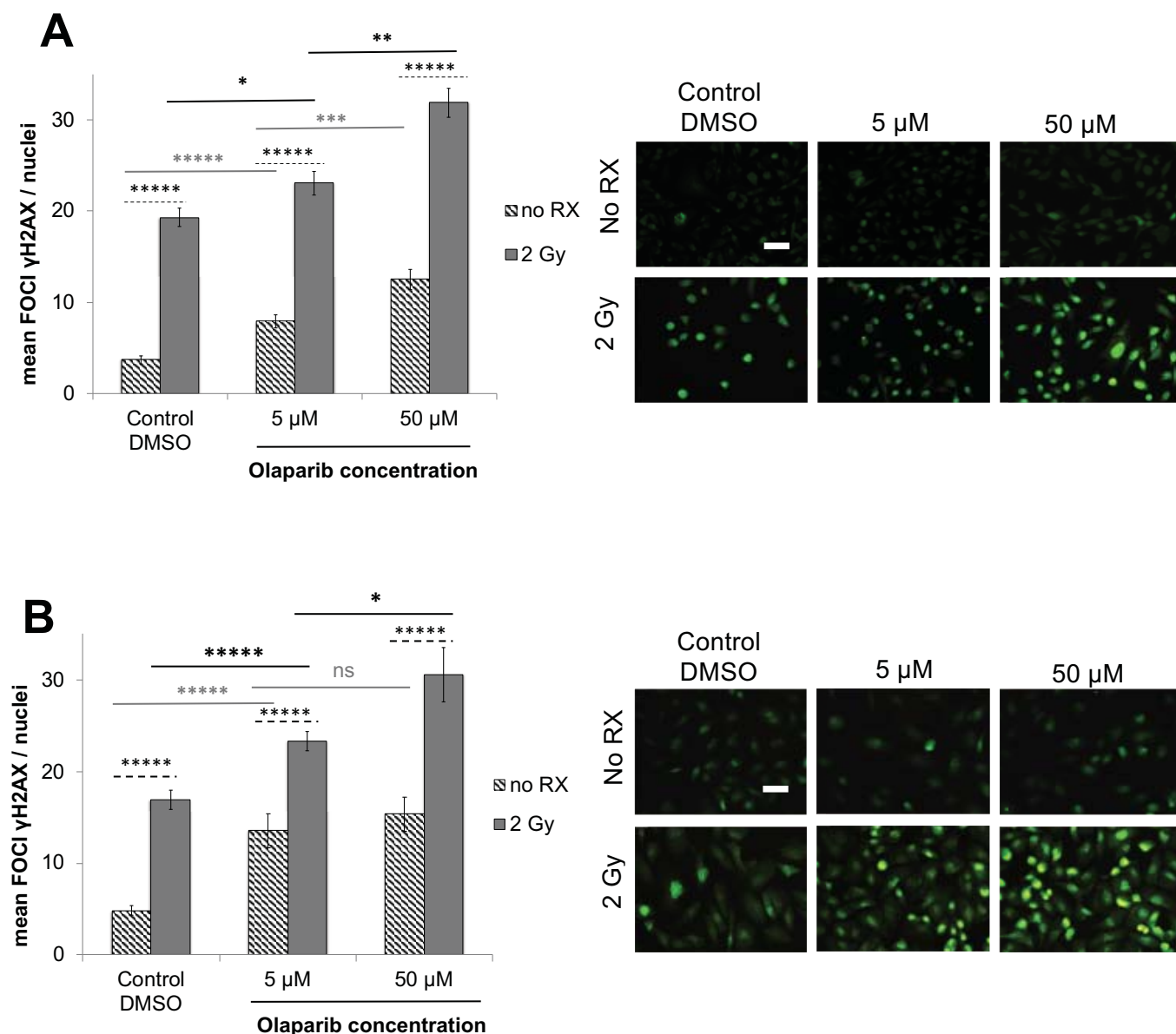


Fig. 1. FOCI gammaH2AX immunofluorescent marking in MDA-MB-231 (A) and SUM1315 (B) cell lines cultured in monolayer after a treatment with 0.1% DMSO (control cells), 5 and 50 μ M Olaparib for 24h alone (no RX) or combined with 2 Gy of radiotherapy (24 and 2 Gy).

*The number of FOCI gammaH2AX per nucleus was determined in >10 fields per condition with ImageJ software. Results are represented as mean number of FOCI gammaH2AX per nuclei with their standard error. A student t-test was performed for statistical comparison of all treatment conditions, where ns = non-significant, * p <0.05, ** p <0.01, *** p <0.001, **** p <0.0001 and ***** p <0.00001. Scale bar = 20 μ m.*

RESULTS

1 Olaparib and 2 Gy irradiation induce increased DNA double strand breaks in MDA-MB-231 and SUM1315 cell lines cultured in monolayer

The number of DNA double-strand breaks per cell induced by a treatment with 5 or 50 μ M Olaparib alone and or then combined with 2 Gy of radiotherapy was determined for both cell lines after 4h, using the foci gammaH2AX immunofluorescent marking (**Fig. 1A et B**).

For MDA-MB-231 cell line (**Fig. 1A**), after a treatment with Olaparib alone, the number of foci per nuclei increased from 4 ± 0.4 for controls, to 8 ± 0.7 for 5 μ M Olaparib ($p=10^{-7}$ compared to controls) and to 13 ± 1.1 for 50 μ M ($p=10^{-12}$ compared to controls). Then, the number of foci per nuclei was determined after a co-treatment of 2 Gy of radiotherapy with or without Olaparib treatment. For irradiated controls, the number of foci H2AX per nuclei increased significantly with 19 ± 1.1 ($p=10^{-26}$ compared to non-irradiated controls). Then, in presence of 5 and 50 μ M Olaparib-irradiated conditions, this parameter increased significantly with 23 ± 1.3 ($p=10^{-20}$ compared to non-irradiated 5 μ M treated cells) and 32 ± 1.6 ($p=10^{-21}$ compared to non-irradiated 50 μ M treated cells), respectively (**Fig. 1A**).

Parallely, for SUM1315 cell line in presence of 5 and 50 μ M Olaparib, the number of foci per nuclei increased significantly with 14 ± 1.8 and 15 ± 1.9 , respectively compared to controls ($p=10^{-5}$ and $p=10^{-7}$, respectively) (**Fig. 1B**). For this cell line, an increase in DNA double-strand-breaks was also detected for irradiated-controls with a mean of 17 ± 1.1 foci per nuclei ($p=10^{-19}$, compared to controls). More, in presence of Olaparib, the number of FOCl increased to 23 ± 1.0 for 5 μ M-irradiated cells (compared to 5 μ M Olaparib alone, $p=10^{-5}$) and to 31 ± 2.9 for 50 μ M-irradiated cells (compared to 50 μ M alone, $p=10^{-5}$) (**Fig. 1B**).

For both cell lines, these results showed that firstly the treatment with Olaparib induced a dose-dependent increase in the number of DNA double strand breaks (DSBs). Secondly, 2 Gy of radiation induced also a significantly increased of DSBs. The combination of Olaparib and in turn of radiotherapy 2 Gy induced higher DSBs in both cell lines, in comparison with the treatment with Olaparib or radiotherapy alone, demonstrating the mechanistically synergistic effect of this co-administration.

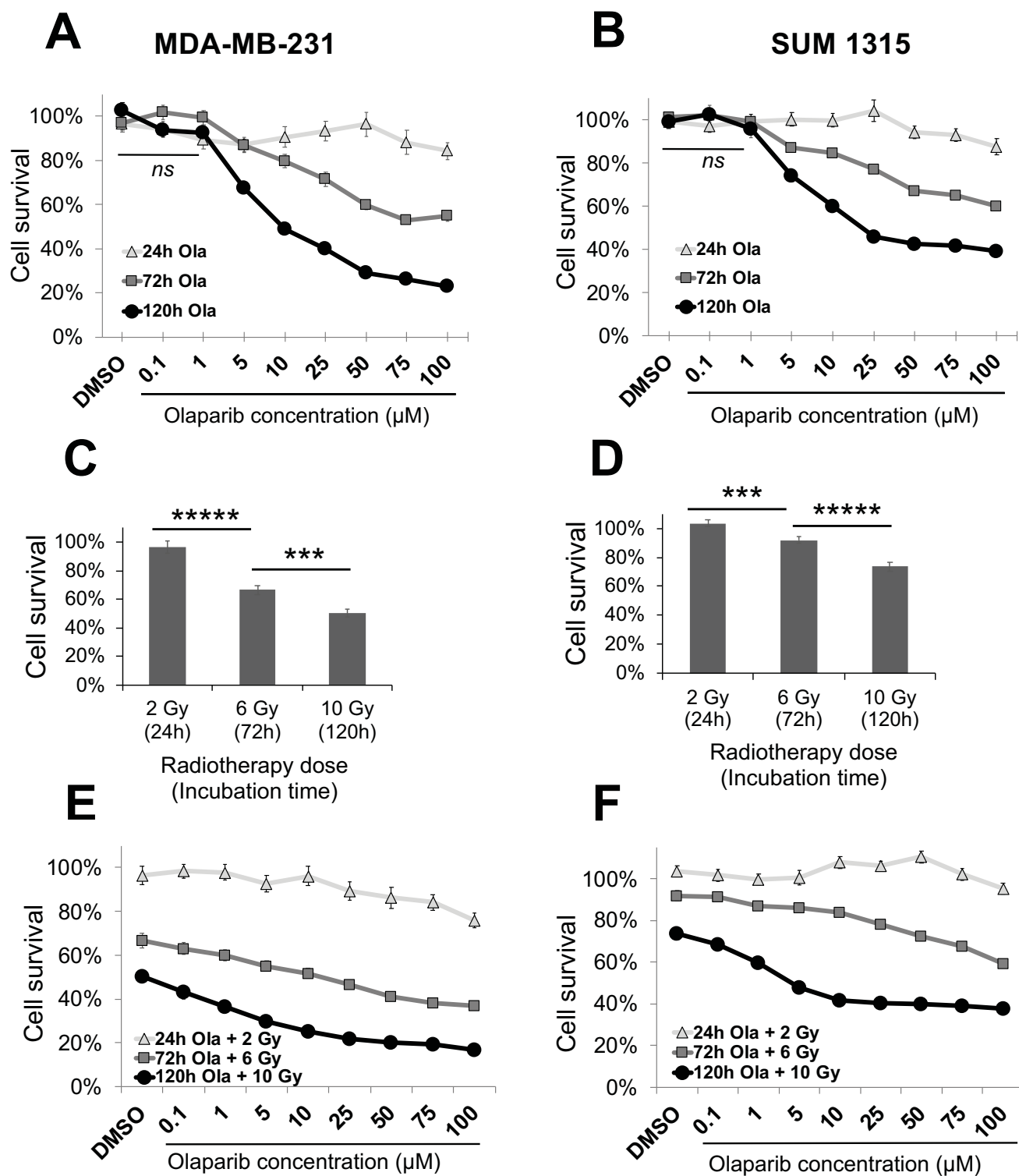


Fig. 2. Cell survival of MDA-MB-231 (A, C, E) and SUM1315 (B, D, F) cell lines after a treatment with Olaparib alone for 24h, 72h and 120h (A, B), or with fractionated radiotherapy alone at 2 Gy (24h), 6 Gy (72h) and 10 Gy (120h) (C, D) or in presence of the co-treatment Olaparib and radiotherapy (E, F).

Cell survival was determined with the SRB survival test. Each intensity value (DO) corresponding to the intensity of cell survival after treatment was normalized by the intensity (DO) of the DMSO controls of each treatment condition. Results are represented as mean % of cell survival with their standard error ($n>30$). A student *t*-test was performed for the statistical comparison of all treatment conditions. Unless for graphs C and D where $***p<0.001$ and $****p<0.00001$, the absence of statistical sign means a significant statistical difference ($p<0.05$) between all treatment endpoints.

2 Cytotoxicity study of Olaparib combined with fractioned radiotherapy on TNBC cell lines in monolayer culture

2.1 Olaparib induces a dose-dependent and time-dependent decrease of MDA-MB-231 and SUM1315 cell lines survival.

The overall survival of cells treated with Olaparib alone (0.1, 1, 5, 10, 25, 50, 75 and 100 μ M for 24, 72 and 120h) was carried out on both MDA-MB-231 and SUM1315 cell lines with the Sulforhodamine B assay (SRB test). Parallely, for both cell models, systematically two controls *i.e.* control cells and cells treated with 0.1% DMSO (DMSO control) were performed in the same experimental conditions. The results were expressed by the percentage of treated cells on 0.1% DMSO controls.

For MDA-MB-231 cell line, the 0.1 % DMSO controls presented same cell survivals as the control cells, with $97\pm4\%$, $97\pm3\%$, $103\pm4\%$ after 24h, 72h and 120h of incubation ($p>0.05$ for all conditions compared to control cells, data not shown) (**Fig. 2A**). After 24h of treatment with Olaparib, the MDA-MB-231 cell survival remained similar to DMSO controls ($97\pm4\%$) for all tested concentrations with $94\pm4\%$ for 0.1 μ M and $84\pm4\%$ for 100 μ M of the anti-PARP ($p>0.05$). After 72h of treatment, no difference in cell survival was detected compared to DMSO controls until 1 μ M Olaparib ($99\pm3\%$, $p>0.05$). Then, cell survival dropped significantly from 5 μ M ($87\pm3\%$, $p=0.03$) to 100 μ M ($55\pm2\%$, $p=10^{-14}$). After 120h of exposition, cell survival decreased significantly for Olaparib concentrations of 1 μ M ($92\pm3\%$, $p=0.04$) to 100 μ M ($23\pm1\%$, $p=10^{-17}$). Similarly, for SUM1315 cell line, the cell survival of the 0.1 % DMSO control conditions were of $99\pm3\%$, $101\pm2\%$ and $99\pm3\%$ after respectively 24h, 72h and 120h of incubation and not significantly different from the survival of control cells for each exposure time ($p>0.05$, data not shown) (**Fig. 2B**). After 24h of treatment with Olaparib, cell survival remained high and non-significantly different from DMSO controls ($99\pm3\%$) until 75 μ M ($93\pm3\%$, $p=0.14$). Only 100 μ M of Olaparib induced a slight decrease in cell survival with $88\pm4\%$ ($p=0.02$). After 72h of treatment, Olaparib induced a significant decrease in cell survival for concentrations of 5 μ M to 100 μ M with $87\pm2\%$ ($p=10^{-7}$) and $60\pm1\%$ ($p=10^{-22}$), respectively. Similarly, after 120h of Olaparib treatment, cell survival dropped significantly compared to DMSO controls ($99\pm3\%$) from 5 μ M ($74\pm3\%$, $p=10^{-7}$) to 100 μ M ($39\pm1\%$, $p=10^{-15}$).

All these results showed a clear Olaparib-dose-dependent effect on both cell lines survival. More, this cytotoxic effect was increased by the time.

2.2 Fractionated radiotherapy induces a dose-dependent decrease in MDA-MB-231 and SUM1315 cell lines survival

The survival of MDA-MB-231 and SUM1315 cells was then determined after a treatment with increasing cumulative doses of radiation of 2 Gy (1 session), 6 Gy (3 sessions) and 10 Gy (5 sessions). The results were expressed by the percentage of irradiated cells on non-irradiated 0.1% DMSO control cells (**Fig. 2C, 2D**).

For MDA-MB-231, no significant difference in cell survival was detected between irradiated control cells and 0.1% DMSO irradiated controls cells ($p > 0.05$, data not shown). After irradiation with 2 Gy, the cell survival remained high with $96 \pm 4\%$ and dropped after 6 Gy and 10 Gy to $67 \pm 3\%$ ($p = 10^{-7}$ compared to 2 Gy) and $50 \pm 3\%$ ($p = 0.0006$ compared to 6 Gy), respectively (**Fig. 2C**). Similarly, for SUM1315 DMSO irradiated-cells, the cell survival was similar to the irradiated-control cells ($p > 0.05$, data not shown). Then, after 2 Gy of radiotherapy, cell survival remained similar to non-irradiated cell controls with $104 \pm 3\%$ and decreased significantly after 6 and 10 Gy with $92 \pm 2\%$ ($p = 0.0006$ compared to 2 Gy) and $73 \pm 3\%$ ($p = 10^{-6}$ compared to 6 Gy), respectively (**Fig. 2D**).

All these results showed that the cytotoxicity of the fractionated radiotherapy regimen (2 Gy per day) on both cell lines was dose-dependent. More, it was not cytotoxic with the low dose of 2 Gy.

2.3 Olaparib combined to fractionated radiotherapy decreases the survival of MDA-MB-231 and SUM1315 cell lines.

Then, the overall survival of MDA-MB-231 and SUM1315 cells first treated with Olaparib (0.1 μM to 100 μM) and then with fractionated radiotherapy (2 Gy for 24h, 6 Gy for 72h or 10 Gy for 120h) was carried out. The results were expressed by the percentage of cell survival (with Olaparib and radiotherapy) compared to the non-irradiated 0.1% DMSO controls (**Fig. 2E, 2F**).

For MDA-MB-231 cell line after 24h of treatment in presence of Olaparib and 2 Gy radiotherapy, cell survival remained similar and non-significantly different to controls for concentrations of 0.1 μM ($98 \pm 3\%$, $p = 0.42$) to 50 μM ($86 \pm 5\%$, $p = 0.13$). Then, from 75 μM to 100 μM , cell survival dropped significantly compared to controls ($76 \pm 3\%$ for 100 μM , $p = 0.0007$) (**Fig. 2E**). After 72h of treatment with Olaparib and 6 Gy radiotherapy, cell survival decreased significantly for all tested Olaparib concentrations with $63 \pm 3\%$ for 0.1 μM ($p = 10^{-10}$) and $37 \pm 2\%$ for 100 μM ($p = 10^{-17}$). Similarly, after 120h of Olaparib treatment and 10 Gy of irradiation, cell

Table 1. Multivariate ANOVA results obtained from the monolayer cell survival dataset.

Experimental parameters	Probability of variance influence	% of variance explanation
Type of cell line (MDA-MB-231, SUM1315)	< 0.0000001	8%
Dose Olaparib (0, 0.1, 1, 5, 10, 25, 50, 75, 100 μ M)	< 0.0000001	14%
Olaparib exposition (24h, 72h, 120h)	< 0.0000001	40%
Radiotherapy Dose (0, 2, 6, 10 Gy)	< 0.0000001	38%

The impact of all experimental parameters (type of cell line, dose of Olaparib, Olaparib time of exposition and dose of radiotherapy) on cell survival variance was analyzed in a multivariate ANOVA analysis, performed with the SEM software (Centre Jean Perrin, FK). The probability of variance influence in the analysis and the percentage of variance explanation were described for each experimental parameter. All parameters explained the survival variance (probabilities inferior to 10^{-6}) and the strongest parameters influencing this variation were the time of Olaparib exposition (40%) and the Radiotherapy dose (38%).

survival dropped significantly for all anti-PARP tested concentrations ($43\pm3\%$ for $0.1\ \mu\text{M}$, $p=10^{-16}$ and $17\pm1\%$ for $100\ \mu\text{M}$, $p=10^{-17}$). For SUM1315 cell line treated with Olaparib for 24h and 2 Gy of radiotherapy, no cytotoxicity compared to controls was detected for any Olaparib concentration, with $95\pm3\%$ after $100\ \mu\text{M}$ ($p=0.29$) (**Fig. 2F**). Then, after 72h of exposition and 6 Gy, cell survival dropped significantly and regularly compared to controls for concentrations of $0.1\ \mu\text{M}$ Olaparib ($91\pm2\%$, $p=10^{-4}$) to $100\ \mu\text{M}$ Olaparib ($59\pm2\%$, $p=10^{-20}$). In contrast, after 120h of treatment and 10 Gy, cell survival dropped drastically for concentrations of $0.1\ \mu\text{M}$ ($68\pm2\%$, $p=10^{-10}$ compared to controls) to $10\ \mu\text{M}$ ($42\pm1\%$, $p=10^{-23}$) and then remained relatively stable for concentrations of $25\ \mu\text{M}$ ($40\pm1\%$) to $100\ \mu\text{M}$ ($38\pm1\%$).

All these results showed that the toxicity effect of a treatment combining Olaparib and fractionated radiotherapy seems to be dependent on the Olaparib administered dose, the dose of radiation, the exposition time and the type of cell line.

2.4 The cytotoxic effect of radiotherapy is potentialized by Olaparib and is more effective in presence of low doses of Olaparib.

In order to determine the impact of each analyzed parameter on cell survival variance, *i.e.* (i) the type of cell line, (ii) the Olaparib concentration, (iii) the Olaparib time of exposition and (iv) the radiotherapy dose, the overall cell survival results from both cell line models were compiled and processed in a multivariate ANOVA analysis (**Table 1 and Fig. 3**). Firstly, the results presented in Table 1 showed a significant influence of all studied parameters on the variation of cell survival with a probability inferior to 10^{-6} for every parameter. Indeed, in our experimental conditions, the cell survival variance was impacted by (i) the time of Olaparib exposition at 40 %, (ii) the radiotherapy dose at 39%, (iii) the Olaparib concentration at 14%, and the type of cell line at 9%. These results suggest clearly that both the duration of Olaparib exposition and the dose of radiotherapy play a major role in the decrease of survival of these TN cell lines.

Secondly, the multivariate ANOVA analysis allowed to determine the impact of the cotreatment combining Olaparib to radiotherapy on cell survival. For this, the impact of radiotherapy dose and the time of Olaparib exposition were studied on all the data of cell survival whatever the type of cell line, after a treatment with Olaparib alone (all concentrations), radiotherapy alone and Olaparib (all concentrations) combined with radiotherapy (**Fig. 3A**). The results showed the same impact on cell survival after 24, 72 and 120h of exposure to Olaparib alone, with $95\pm1\%$, $80\pm1\%$ and $62\pm2\%$ or after radiotherapy alone, with 2 Gy at $100\pm2\%$, 6 Gy at $81\pm3\%$ and 10 Gy at $62\pm3\%$, respectively. In contrast, the combination of Olaparib and radiotherapy treatment caused a significant cell survival decrease from 6 Gy and 72h exposure with $64\pm1\%$,

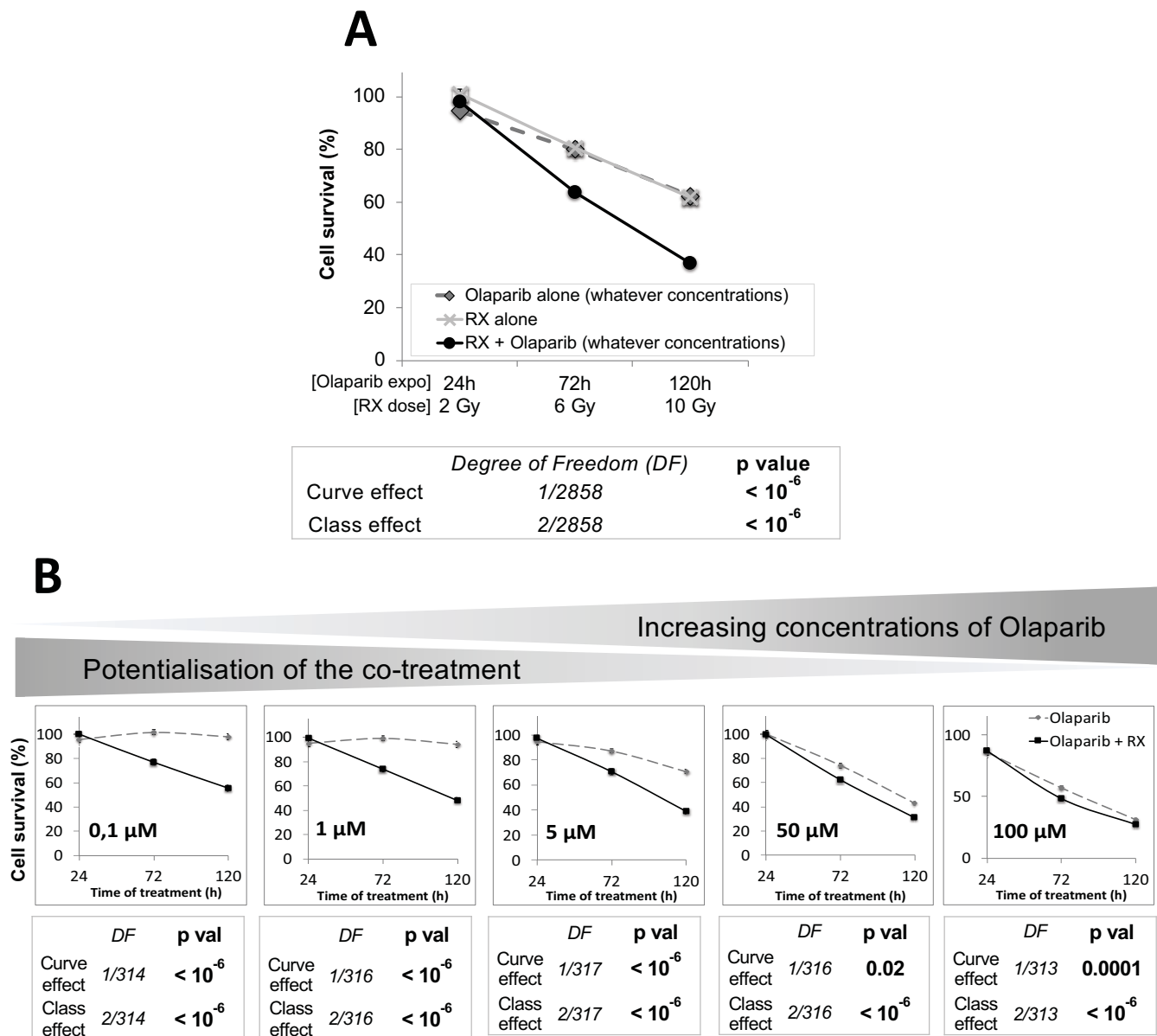


Fig. 3. ANOVA analysis of monolayer cell culture sensitivity after the co-treatment: the cytotoxic effect of radiotherapy is potentialized by Olaparib and is more effective in presence of low doses of Olaparib.

The graph (A) describes the decrease in the cell survival of both cell lines (MDA-MB-231 and SUM1315) by the time, according to (i) the Olaparib treatment alone (all concentrations $\geq 0.1 \mu\text{M}$ for each endpoint), (ii) the treatment with fractioned radiotherapy alone or (iii) the co-treatment with Olaparib (all concentrations $\geq 0.1 \mu\text{M}$) and fractioned radiotherapy.

The second set of graphs (B) describes the decrease of cell survival of both cell lines by the time according to (i) the Olaparib treatment alone after each concentration of $0.1 \mu\text{M}$, $1 \mu\text{M}$, $5 \mu\text{M}$, $50 \mu\text{M}$ or $100 \mu\text{M}$ and (ii) the co-treatment Olaparib (same concentrations) with fractioned radiotherapy.

For A and B, the curve effect (= significant decrease in cell survival by the time for a same condition) and the class effect (= significant difference in cell survival for a same incubation time between each condition) were determined by the ANOVA analysis, associated with their degree of freedom (= number of data of each analysis).

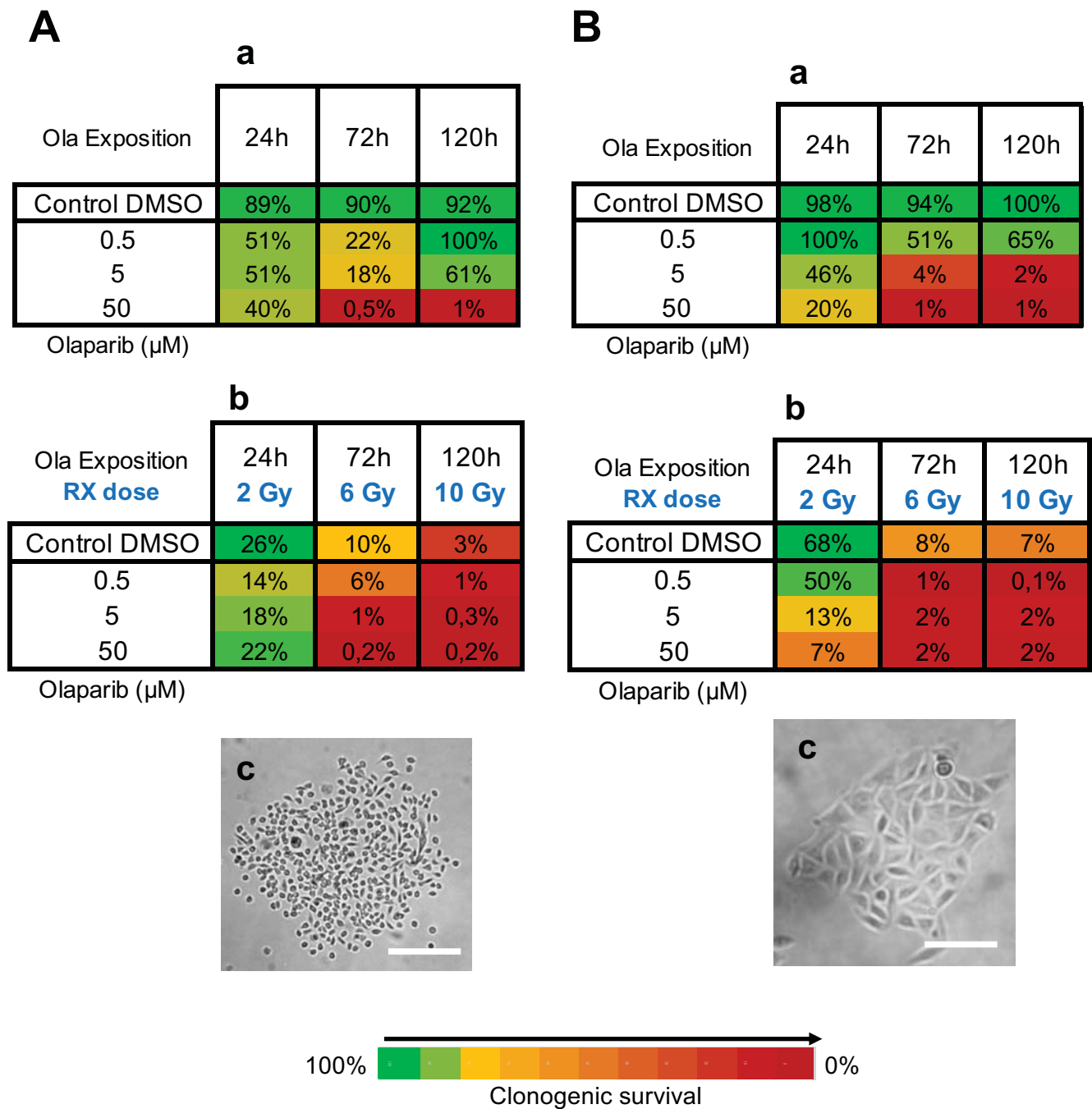
which kept decreasing after 10 Gy and 120h exposure with $37\pm1\%$. These results showed clearly that Olaparib combined with radiotherapy induced a significant decrease in cell survival compared to radiotherapy alone, or Olaparib alone, and this, independently of the Olaparib concentration or the type of cell line. Indeed, the curve effect and class effect were both inferior to 10^{-6} in the ANOVA analysis.

Thirdly, the impact of each Olaparib tested concentration on cell survival variance was determined. For this, the cell survival of both cell lines treated with each Olaparib concentration (0.1 μM to 100 μM) was analysed in the ANOVA analysis for 24h, 72h and 120h in presence of Olaparib alone or after the combined regimen with fractionated radiotherapy (2 to 10 Gy) (**Fig. 3B**). This analysis showed a strong decrease in survival for cells treated with 0.1 to 5 μM Olaparib for 120h and 10 Gy of radiotherapy compared to 120h Olaparib treatment alone. Indeed, cell survival was of $56\pm3\%$ vs $98\pm2\%$ for 0.1 μM , of $48\pm2\%$ vs $94\pm3\%$ for 1 μM and of $39\pm2\%$ vs $71\pm2\%$ for 5 μM , respectively. More, the curve effect probabilities for each dose were systematically inferior to 10^{-6} . In contrast, with higher Olaparib concentrations of 50 and 100 μM , cell survival remained similar at 120h between a treatment with Olaparib alone or combined with 10 Gy radiotherapy, with $30\pm2\%$ vs 36 ± 2 for 50 μM and 27 ± 2 vs $31\pm2\%$ for 100 μM , with curve effect probabilities of 0.02 and 0.0001, respectively.

These results showed that the co-treatment Olaparib and radiotherapy was more efficient than a treatment with Olaparib alone, or radiotherapy alone. Thus, this analysis showed that the beneficial of the co-treatment (Olaparib + RX) compared to Olaparib treatment alone was more important with low Olaparib concentrations.

3 The co-treatment decreases the clonogenic repopulation potential of MDA-MB-231 and SUM1315 cell lines cultured in monolayer

In order to determine the long-term effect of the co-treatment on the repopulation potential of cells by the formation of cell colonies (**Fig. 4Ac, 4Bc**), the clonogenic survival of cells treated with Olaparib alone (concentrations of 0.5, 5 or 50 μM for 24, 72 and 120h) or concomitantly with radiotherapy (2, 6 and 10 Gy, respectively) was determined for both MDA-MB-231 (**Fig. 4A**) and SUM1315 (**Fig. 4B**) cell lines cultured in monolayer. For MDA-MB-231 cell line, the clonogenic survival of the 0.1% DMSO controls was not significantly different from the controls with $89\pm8\%$, $90\pm6\%$, $92\pm4\%$ after 24h, 72h and 120h, respectively ($p>0.05$) (**Fig. 4Aa**). After 24h of treatment with Olaparib (**Fig. 4Aa**), the clonogenic survival of cells decreased



Standard errors are [$< 10\%$] for every treatment condition ($n > 3$)

Fig. 4. Clonogenic repopulation potential of MDA-MB-231 (A) and SUM1315 (B) cell lines cultured in monolayer, after a treatment with 0.5, 5 and 50 μM Olaparib alone for 24h, 72, or 120h (a), or combined with fractioned radiotherapy of 2 to 10 Gy (b).

For each treatment condition, the percentage of clonogenic survival was determined by the ratio of the survival fraction of treated cells on the survival fraction of 0.1% DMSO control cells. Results are represented on a Heatmap. Standard errors are [$< 10\%$] for every treatment condition ($n > 3$). (c) Photographs of clonogenic populations of MDA-MB-231 and SUM1315, magnification = 40X, scale bar = 50 μm .

significantly than controls and then remained similar for the both 0.5 and 5 μM Olaparib concentrations, with $51\pm 2\%$ and $51\pm 4\%$, respectively ($p=0.04$, $p=0.01$). This drop continued even slightly to $40\pm 3\%$ in presence of 50 μM Olaparib ($p=0.03$, than controls). Similarly, after 72h of Olaparib exposition (**Fig. 4Aa**), the clonogenic survival decrease was higher for 0.5 and 5 μM of this anti-PARP with $22\pm 2\%$ and $18\pm 1\%$ respectively ($p=10^{-6}$ et $p=10^{-6}$). Then, this parameter decreased drastically to $0.5\pm 0.1\%$ ($p=10^{-6}$) in presence of 50 μM Olaparib. In contrast, after 120h of treatment (**Fig. 4Aa**), the clonogenic survival increased significantly again, for all Olaparib concentrations with $100\pm 3\%$, $61\pm 14\%$ and $1\pm 0.1\%$ respectively ($p=0.05$, $p=0.20$ and $p=0.007$, respectively compared to 72h). These results suggest that after a longtime exposition to Olaparib (120h), the potential clonogenic of MDA-MB-231 cell lines may be again restarted.

Then, the effect of radiotherapy alone was determined on the MDA-MB-231 clonogenic survival (**Fig.4Ab**). The results showed a same clonogenic survival for the irradiated controls and 0.1% DMSO irradiated controls cells. Indeed, it was $26\pm 7\%$ with 2 Gy of radiotherapy after 24h. Then, this parameter dropped regularly to $10\pm 2\%$ with 6 Gy after 72h and to $3\pm 1\%$ with 10 Gy after 120h ($p<0.05$). These results showed that fractioned radiotherapy induced a clear potential clonogenic decrease very effective at 10 Gy after 120h exposure. The clonogenic survival of cells was then followed in time after a combined Olaparib and radiotherapy treatment, in the same experimental conditions (**Fig. 4Ab**). In presence of Olaparib for 24h and 2 Gy of radiotherapy, the clonogenic survival decreased significantly compared to 2 Gy radiotherapy with $14\pm 1\%$ after 0.5 μM ($p=0.03$). Then, this parameter remained stable for 5 μM and 50 μM with $18\pm 7\%$ ($p=0.35$) and $22\pm 1\%$ ($p=0.47$), respectively. After 72h of Olaparib treatment and 6 Gy, the clonogenic survival decreased drastically compared to 6 Gy radiotherapy alone for 5 and 50 μM Olaparib concentrations with $1\pm 0.1\%$ and $0.5\pm 0.1\%$ respectively ($p=10^{-5}$ and $p=0.01$, respectively). Similarly, after 120h of Olaparib and 10 Gy of radiotherapy, the clonogenic survival decreased significantly for all Olaparib concentrations compared to 10 Gy radiotherapy with $1\pm 0.5\%$, $0.3\pm 0.1\%$ and $0.2\pm 0.1\%$, respectively ($p=0.03$, $p=0.003$ and $p=0.003$, respectively). For MDA-MB-231 cell line, a cotreatment with all tested Olaparib concentrations and fractioned 6 or 10 Gy radiotherapy reduced strongly their potential of clonogenic repopulation.

For SUM1315 cell line, the 0.1% DMSO controls after 24h, 72h and 120h incubation presented same clonogenic survivals as the controls with $98\pm 8\%$, $94\pm 2\%$ and $100\pm 18\%$, respectively ($p>0.05$ for all conditions) (data not shown). After 24h of treatment with Olaparib alone (**Fig. 4Ba**), the clonogenic survival remained similar to 0.1% DMSO control for 0.5 μM ($100\pm 15\%$) and decreased progressively after exposure to 5 μM with $46\pm 16\%$ ($p=0.02$) and 50 μM with

20±1% (p=0.03). After 72h of Olaparib treatment, the clonogenic survival decreased first slightly to 51±6% (p=0.007, compared to control) with 0.5 µM and then, drastically to 4±1% (p=0.005) and 1±0.1% (p=0.001), with 5 and 50 µM, respectively. After 120h of treatment with Olaparib, the clonogenic survival increased for 0.5 µM at 65±1% (p=0.008) and then, decreased dramatically for 5 and 50 µM with 2±1% and 1±0.1%, respectively (p=0.001 and p=0.001). On the drop in clonogenic survival of SUM1315 cell line, Olaparib would have a dose and time dependent action.

The action of fractioned radiotherapy was then analyzed on survival clonogenic of SUM1315 cell line, in time (**Fig. 4Bb**). The results showed that clonogenic survivals of 0.1% DMSO irradiated controls were similar to irradiated controls cells for all radiotherapy doses with 68±14%, 8±2% and 7±1%, after 2, 6 and 10 Gy respectively (p>0.05 for all conditions). These results showed that 6 and 10 Gy fractioned radiotherapy induced a similar drastically decrease of clonogenic potential on this cell line.

In addition, the analysis of the co-treatment 0.5 µM Olaparib and 2 Gy radiotherapy for 24h (**Fig. 4Bb**) showed a clonogenic survival of 50±5% (p=0.40, compared to radiotherapy 2 Gy). Then, this parameter decreased strongly to 13±2% and 7±0.1% in presence of 5 and 50 µM Olaparib / 2Gy radiotherapy, respectively (p=0.04 and p=0.03). After 72h and 6 Gy, clonogenic survival dropped strongly with 0.5 to 50 µM Olaparib with 1.4±0.4% and 2±0.1%, respectively (p=0.01 and p=0.02 compared to 6 Gy controls). Similarly, after 120h and 10 Gy, the clonogenic survival dropped compared to controls for all concentrations (with 2±1% for 50 µM). For SUM1315 cell line, a cotreatment with all tested Olaparib concentrations and fractioned 6 or 10 Gy radiotherapy reduced significantly their clonogenic repopulation potential.

All these results confirmed the time-dependent effect of Olaparib alone on the inhibition of the clonogenic potential of MDA-MB-231 and SUM1315 cell lines. More, this effect was drastically potentialized by the co-treatment with radiotherapy. Finally, the co-treatment with low dose of Olaparib and fractioned radiotherapy of 10 Gy induces very low clonogenic potential inferior to 1% indicating a nearly eradicated repopulation potential of these cell lines.

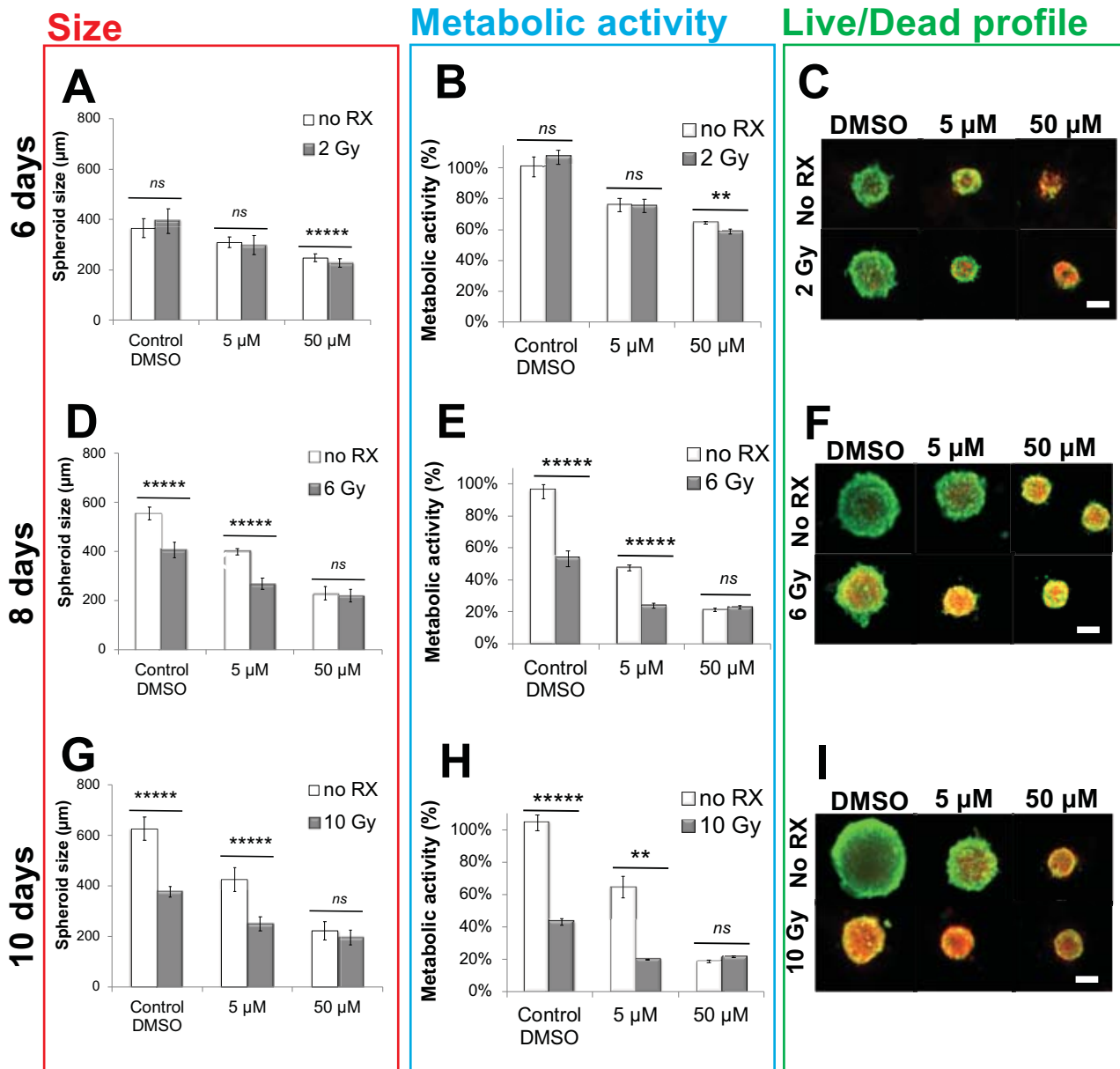


Fig. 5. MDA-MB-231 spheroids size (A, D, G), metabolic activity (B, E, H) and live/dead profile (C, F, I) monitoring after a treatment with Olaparib alone (5 and 5 μM) for 6, 8 and 10 days, with fractionated radiotherapy alone (2 Gy for 6 days, 6 Gy for 8 days and 10 Gy for 10 days) or with the combined treatment Olaparib and fractionated radiotherapy.

(A, D, G) MDA-MB-231 spheroid size evolution after a treatment with Olaparib and/or fractionated radiotherapy for 6 days (A), 8 days (D) and 10 days (G): spheroids were imaged with Cytation3MV plate reader and their size (μm) was measured with Gen 5.03 software (Biotek). Results are represented as mean spheroid size ($n>30$) with their standard errors.

(B, E, H) MDA-MB-231 cell metabolic activity in spheroids with resazurin test after a treatment with Olaparib and/or fractionated radiotherapy for 6 days (B), 8 days (E) and 10 days (H): Corrected fluorescence intensity of resorufin (ex/em $\lambda 576/\lambda 584$ nm) was measured after 15h incubation with 60 μM resazurin in PBS. The fluorescence intensity of treated spheroids ($n>15$) was normalized by the fluorescence intensity of each associated 0.1% DMSO control spheroids. Results are expressed as mean metabolic activity (%) of each treatment conditions with their standard errors. A student t-test was performed for the statistical comparison of all treatment conditions and the p value is represented in the graphs as ns = non-significant, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$ and ***** $p<0.00001$.

(C, F, I) Cell viability/mortality profile in spheroids with LiveDead® imaging after treatment with Olaparib and/or fractionated radiotherapy for 6 days (C), 8 days (F) and 10 days (I): Each image is representative of three acquisitions of the same treatment condition. Images were taken with Cytation3MV where green markings correspond to calcein-AM penetration (viable cells) and red markings correspond to ethidium homodimer-1 cell penetration (dead cells). Scale bar = 200 μm

4 The co-treatment Olaparib followed with fractioned radiotherapy induces decreased cell proliferation and cell metabolic activity on both triple negative MDA-MB-231 and SUM1315 spheroids

The analysis of spheroid cell proliferation and metabolic activity after a treatment with Olaparib alone (5 or 50 μM) for 6, 8 and 10 days or concomitantly with 2 Gy (6 days), 6 Gy (8 days) or 10 Gy (10 days) of radiotherapy, respectively, was assessed on both MDA-MB-231 and SUM1315 cell lines. In parallel, two controls, *i.e.* control spheroids and spheroids treated with 0.1% DMSO were performed. Cell proliferation was analyzed by a spheroid size measurement, cell metabolic activity by the resazurin test and spheroid viability and mortality profile by the Live/Dead fluorescent marking. For these experiments, all analyses were carried out at the end of each treatment exposition time with the Cytation3MV plate reader.

4.1 MDA-MB-231 spheroids

4.1.1 Size, metabolic activity and live/dead profile change in MDA-MB-231 spheroids at day 6 after a treatment with increasing Olaparib concentration and 2 Gy radiotherapy

The size and metabolic activity of MDA-MB-231 spheroids at 6 days (Fig. 5A, B, C) for 0.1% DMSO control ($362 \pm 16 \mu\text{m}$ and $101 \pm 7\%$) and after a radiotherapy with 2 Gy ($386 \pm 65 \mu\text{m}$ and $107 \pm 5\%$) remained similar, respectively ($p=0.33$ and $p=0.55$). In the condition of treatment with 5 μM Olaparib alone and combined 5 μM Olaparib and 2 Gy radiotherapy, spheroid size ($309 \pm 21 \mu\text{m}$ and $298 \pm 37 \mu\text{m}$) and metabolic activity ($76 \pm 4\%$ and $76 \pm 4\%$) decreased significantly compared to controls, respectively ($p=10^{-10}$ and $p=10^{-11}$ for size and $p=0.01$ and 0.0002 for metabolic activity). In contrast, for both parameters, no significant difference was detected between condition of treatment with Olaparib alone and Olaparib / radiotherapy ($p=0.18$ and $p=0.97$). Similarly, in presence of 50 μM Olaparib and co-treatment 50 μM Olaparib / radiotherapy, spheroid size ($248 \pm 15 \mu\text{m}$ and $227 \pm 16 \mu\text{m}$) and metabolic activity ($64 \pm 1\%$ and $59 \pm 2\%$) dropped significantly than controls, respectively ($p=10^{-22}$ and $p=10^{-23}$ for size and $p=0.003$ and 10^{-7} for viability). Live/dead profile (**Fig. 5C**) confirmed also the presence of a majority of dead cells (red marked cells) in spheroids in this condition. However, for both parameters, this decrease was higher in condition of the co-treatment than Olaparib alone ($p=10^{-6}$ for size and $p=0.009$ for metabolic activity).

4.1.2 Size, metabolic activity and live/dead profile change in MDA-MB-231 spheroids at day 8 after a treatment with increasing Olaparib concentrations and fractionated 6 Gy radiotherapy

After a fractionated 6 Gy radiation treatment (2 Gy by day) and 8 days of incubation, MDA-MB-231 spheroids size decreased significantly with $407 \pm 40 \mu\text{m}$ in comparison with 0.1% DMSO control spheroids with $563 \pm 20 \mu\text{m}$ that had proliferated ($p=10^{-29}$) (**Fig. 5 D**). Similarly, metabolic activity (**Fig. 5 E, F**) dropped significantly in this condition to $53 \pm 5\%$ with the presence of many dead cells (red marking) against controls spheroids ($96 \pm 4\%$ with green spheroids, $p=10^{-5}$). In presence of 5 μM Olaparib alone (**Fig. 5 D, E, F**), these two parameters decreased again significantly (size $399 \pm 13 \mu\text{m}$ and metabolic activity $47 \pm 2\%$ of smaller green spheroids) than spheroid controls ($p=10^{-34}$ for size and $p=10^{-7}$ for metabolic activity). In contrast, this drop was clearly stronger when the Olaparib was combined with 6 Gy radiotherapy. Indeed, small yellow / red (dead cells in majority) spheroids exhibited a size of $270 \pm 23 \mu\text{m}$ and a metabolic activity of $24 \pm 2\%$ both inferior to the condition with Olaparib alone ($p=10^{-32}$ and $p=10^{-5}$) (**Fig. 5 D, E, F**). Size and metabolic activity in MDA-MB-231 spheroids treated with 50 μM Olaparib alone or combined treatment 50 μM Olaparib and 6 Gy radiotherapy remained similar ($230 \pm 27 \mu\text{m}$ and $222 \pm 24 \mu\text{m}$ for size and $21 \pm 1\%$ and $23 \pm 1\%$ for metabolic activity, respectively, $p=0.16$ and $p=0.20$) (**Fig. 5 D, E**). More, these latest values remained close to those of treatment conditions with 5 μM Olaparib and 6 Gy of radiotherapy ($p=0.56$ for metabolic activity). Live/dead profile confirmed these data showing the small spheroids with majority of dead cells (yellow/red marking) for all these conditions (**Fig. 5 F**).

4.1.3 Size, metabolic activity and live/dead profile change in MDA-MB-231 spheroids at day 10 after a treatment with increasing Olaparib concentration and fractionated 10 Gy radiotherapy

After 10 Gy fractionated radiotherapy at day 10 (**Fig. 5 G, H, I**), small spheroids with the size of $381 \pm 23 \mu\text{m}$ presenting yellow / red marking cells and a metabolic activity of $43 \pm 2\%$ were detected. These two parameters were significantly lower than controls exhibiting big spheroids with size of $658 \pm 31 \mu\text{m}$ and metabolic activity of $105 \pm 5\%$ ($p=10^{-29}$ and $p=10^{-7}$, respectively). 5 μM Olaparib after 10 days of treatment induced also a clear decrease of size with $426 \pm 48 \mu\text{m}$ ($p=10^{-26}$) and metabolic activity of $65 \pm 7\%$ than controls ($p=10^{-9}$). However, the decrease of these two parameters was accentuated with 10 Gy radiotherapy engendering small spheroids containing a large number of dead cells (yellow/red marking) with size of $249 \pm 27 \mu\text{m}$ and metabolic activity of $20 \pm 0.4\%$ than Olaparib 5 μM alone ($p=10^{-23}$ for size and $p=0.001$ for viability) (**Fig. 5 G, H, I**). In contrast, only a slight difference was detected between spheroids treated with 50 μM Olaparib and these with 50 μM Olaparib / 10 Gy radiotherapy with a size of

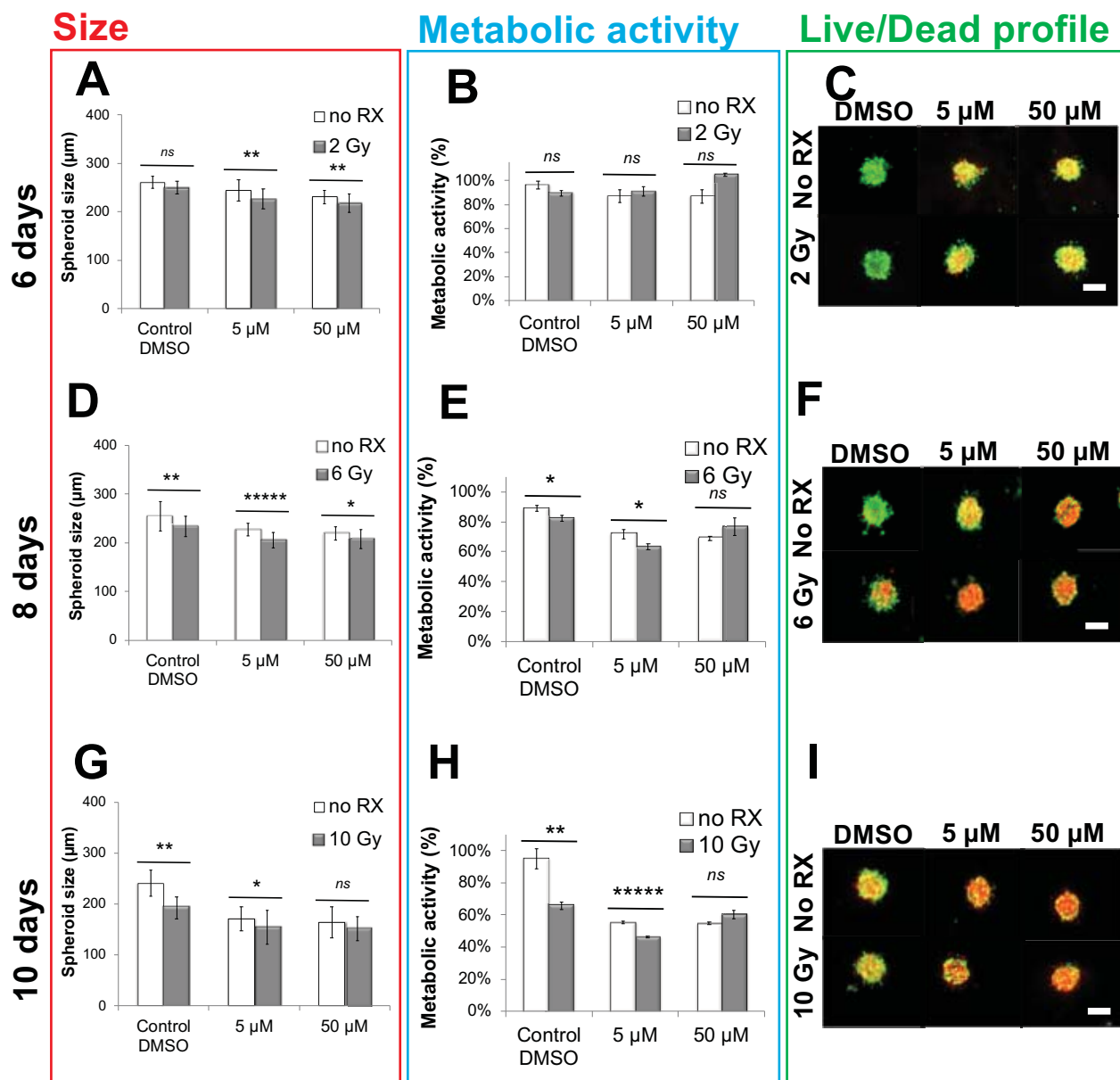


Fig. 6. SUM1315 spheroids size (A, D, G), metabolic activity (B, E, H) and live/dead profile (C, F, I) monitoring after a treatment with Olaparib alone (5 and 5 μM) for 6, 8 and 10 days, with fractionated radiotherapy alone (2 Gy for 6 days, 6 Gy for 8 days and 10 Gy for 10 days) or with the combined treatment Olaparib and fractionated radiotherapy.

(A, D, G) SUM1315 spheroid size evolution after a treatment with Olaparib and / or fractionated radiotherapy for 6 days (A), 8 days (D) and 10 days (G): spheroids were imaged with Cytation3MV plate reader and their size (μm) was measured with Gen 5.03 software (Biotek). Results are represented as mean spheroid size ($n > 30$) with their standard errors.

(B, E, H) SUM1315 cell metabolic activity in spheroids with resazurin test after a treatment with Olaparib and / or fractionated radiotherapy for 6 days (B), 8 days (E) and 10 days (H): Corrected fluorescence intensity of resorufin (ex/em $\lambda 576/\lambda 584$ nm) was measured after 15h incubation with 60 μM resazurin in PBS. The fluorescence intensity of treated spheroids ($n > 15$) was normalized by the fluorescence intensity of each associated 0.1% DMSO control spheroids. Results are expressed as mean metabolic activity (%) of each treatment conditions with their standard errors. A student *t*-test was performed for the statistical comparison of all treatment conditions and the *p* value is represented in the graphs as ns = non-significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ and ***** $p < 0.00001$.

(C, F, I) Cell viability / mortality profile in spheroids with LiveDead® imaging after treatment with Olaparib and / or fractionated radiotherapy for 6 days (C), 8 days (F) and 10 days (I): Each image is representative of three acquisitions of the same treatment condition. Images were taken with Cytation3MV where green markings correspond to calcein-AM penetration (viable cells) and red markings correspond to ethidium homodimer-1 cell penetration (dead cells). Scale bar = 200 μm

222±36 and 197±29 µm and a metabolic activity of 19±1% and 21±0.4%, respectively (p=0.008 for size and p=0.005 for viability) (**Fig.5 G, H**). For these both conditions, in small spheroids the presence a marking yellow / red cells demonstrating the presence of many dead cells was detected (**Fig. 5I**).

All these results showed that firstly, in MDA-MB-231 spheroids, a co-treatment with Olaparib / radiotherapy induced a clear decrease of size and metabolic activity rates, whatever the dose of Olaparib (5 or 50 µM) or radiotherapy intensity (2, 6 and 10 Gy). However, the best efficacy of the co-treatment seems to be obtained in spheroids exposed to 5 µM Olaparib and 6 Gy or 10 Gy of radiotherapy.

4.2 SUM1315 spheroids

4.2.1 Size, metabolic activity and live/dead profile change in SUM1315 spheroids after 6 days of treatment with Olaparib and fractioned radiotherapy of 2 Gy

The size and metabolic activity of SUM1315 0.1% DMSO control spheroids with 261±1 µm and 96±3% remained similar to the 2 Gy-irradiated 0.1% DMSO controls with 250±11 µm and 89±2%, respectively (p=0.12 and p=0.21) (**Fig. 6A, B**). More, no difference in Live/Dead green markings was detected between the two conditions (**Fig. 6C**). Then, in presence of 5 µM coupled to 2 Gy and 5 µM Olaparib alone, spheroid size decreased slightly compared to controls with 227±19 µm (p=10⁻⁶) and 245±22 µm (p=0.0006) and was significantly different between both conditions (p=0.002). In contrast, spheroid metabolic activity of 5 µM Olaparib / radiotherapy condition was similar to 5 µM Olaparib (91±4% and 87±5%, respectively, p=0.60). In presence of 50 µM Olaparib and 50 µM Olaparib / radiotherapy, spheroid size (231±14 µm and 218±19 µm) dropped slightly than controls (p=10⁻¹³ and p=10⁻¹⁰, respectively) and was again significantly different between the two conditions (p=0.003). Similarly, spheroid metabolic activity after 50 µM Olaparib and 2 Gy treatment remained similar to 50 µM Olaparib alone, with 100±5% vs 87±5% (p=0.05). More, Live/Dead profiles presented similar markings (yellow/red cells) between 5 and 50 µM treated spheroids, with or without radiotherapy (**Fig. 6C**).

4.2.2 Size, metabolic activity and live/dead profile change in SUM1315 spheroids after 8 days of treatment with increasing concentrations of Olaparib and fractioned 6 Gy radiotherapy

The SUM1315 control spheroids treated with 6 Gy of fractioned radiotherapy for 8 days of incubation presented significantly decreased size and metabolic activity (233±22 µm and

82±2%) than controls with 254±30 µm and 89±2% (p=0.007 and p=0.04, respectively) (**Fig. 6 D, E**). These results were confirmed by the presence of increased red cells in the live/dead markings of these irradiated spheroids (**Fig. 6 F**). In presence of 5 µM of Olaparib alone or combined to 6 Gy radiotherapy (**Fig. 6 D, E**), spheroid size (227±13 µm and 205±16 µm, respectively with p=0.0002 and p=10⁻⁷) and metabolic activity (72±3% and 63±2%, respectively with p=0.0005 and p=0.001) decreased slightly but significantly than controls. More, the co-treated spheroids presented sizes and metabolic activities inferior to the Olaparib-treated spheroids alone (p=10⁻⁷ and p=0.03, respectively), as well as increased dead cells in the live/dead test (**Fig. 6F**). With 50 µM of Olaparib alone, spheroid size and metabolic activity dropped significantly than controls with 219±13 µm and 69±1%, respectively (p=10⁻⁷ and p=10⁻⁶, respectively). This drop was also detected in the live/dead markings with the presence of dead cells in 50 µM treated spheroids. Then, 50 µM and 6 Gy radiotherapy spheroids exhibited decreased size of 208±20 µm compared to 6 Gy controls (p=10⁻⁵) and similar metabolic activity of 77±6% (p=0.17). More, the co-treated spheroids presented sizes inferior to the Olaparib-treated spheroids (p=0.01), similar metabolic activity (p=0.37) and similar viability/mortality test showed similar spheroid profiles (**Fig. 6F**).

4.2.3 Size, metabolic activity and live/dead profile change in SUM1315 spheroids after 10 days of treatment with increasing concentrations of Olaparib and fractioned 10 Gy radiotherapy

After 10 Gy of fractioned radiotherapy and 10 days of incubation, SUM1315 irradiated spheroids presented decreased size and metabolic activity than 0.1% DMSO controls with 192±16 µm and 66±3% against 240±7 µm (p=0.009) and 95±6%, respectively (p=0.001) (**Fig. 6 G, H**). Similarly, Live/dead test showed increased yellow / red cells in the irradiated spheroids (**Fig. 6I**). In presence of 5 µM Olaparib and 10 Gy radiotherapy, spheroid size and metabolic activity decreased again significantly (154±33 µm and 46±0.4%) compared to 5 µM Olaparib alone with 170±24 µm and 55±1% (p=0.04 for size and p=10⁻⁷ for metabolic activity) and to controls (p=10⁻⁵ and p=10⁻⁶). Parallely, the dead cells (red markings) increased in co-treated spheroids compared to Olaparib alone (**Fig. 6 I**). In presence of 50 µM Olaparib with or without 10 Gy radiotherapy, no significant difference was detected in spheroids size with 164±30 for the co-treatment and 152±23 µm for Olaparib alone (p=0.10). Similarly, no difference in metabolic activity and live/dead red markings was detected between the two treatment conditions with 60±3% and 55±1%, respectively (p=0.051) (**Fig. 6H, I**). All these results showed that SUM1315 spheroids are sensible at long-term to the co-treatment Olaparib / radiotherapy in terms of spheroid size and metabolic activity decrease. More, the best efficacy

Table 2. General results of the multivariate ANOVA obtained from the spheroid metabolic activity dataset.

Experimental parameters	Probability of variance influence	% of variance explanation
Type of cell line (MDA-MB-231, SUM1315)	< 0.0000001	5%
Dose Olaparib (0, 5, 50 µM)	< 0.0000001	13%
Olaparib exposition (6, 8, 10 days)	< 0.0000001	37%
Radiotherapy Dose (0, 2 Gy, 6 Gy, 10 Gy)	< 0.0000001	42%

The impact of all experimental parameters (type of cell line, dose of Olaparib, Olaparib time of exposition and dose of radiotherapy) on spheroid metabolic activity variance was analyzed in a multivariate ANOVA analysis, performed with the SEM software (Centre Jean Perrin). The probability of variance influence in the analysis and the percentage of variance explanation were described for each experimental parameter. All parameters explained the survival variance (probabilities inferior to 10^{-6}) and the strongest parameters influencing this variation were the time of Olaparib exposition (37%) and the radiotherapy dose (42%).

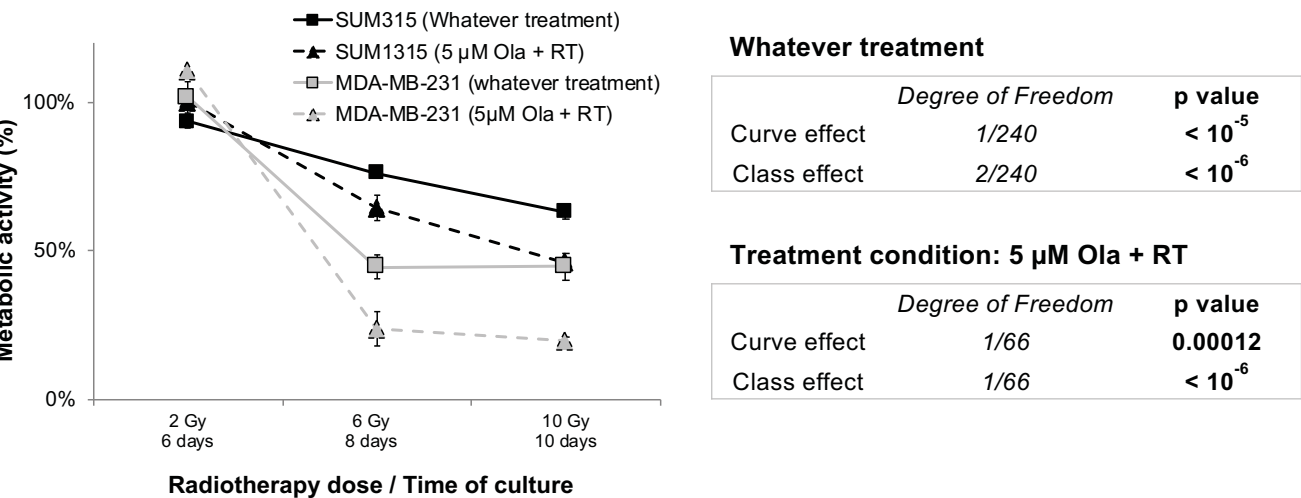


Fig. 7. ANOVA analysis of spheroids sensitivity to the co-treatment with Olaparib and 2 to 10 Gy of fractioned radiotherapy: the decrease in spheroid metabolic activity is potentialized with 5 µM Olaparib and heterogenous between the two MDA-MB-231 and SUM1315 cell lines.

ANOVA analysis of metabolic activity of spheroid from each cell line by the time whatever dose of Olaparib (0, 5 and 50) and whatever type of treatment (no treatment, RX, Ola, ou RX+Ola) and after 5 µM Olaparib and fractioned radiotherapy.

of the co-treatment seems to be obtained in spheroids exposed to 5 μ M Olaparib and 10 Gy of radiotherapy.

5 The cytotoxic effect of the co-treatment Olaparib / radiotherapy in triple negative spheroids is cell line dependant and is effective with low doses of Olaparib

All data obtained from the resazurin metabolic activity test on MDA-MB-231 and SUM1315 spheroids were compiled and processed in a multivariate ANOVA analysis (**Table 2, Fig. 7**). First, the percentage of metabolic activity variance explanation was determined for each experimental variable, *i.e.* the type of cell line (MDA-MB-231 or SUM1315), the dose of Olaparib (none, 5 or 50 μ M), the Olaparib exposition time (6, 8 or 10 days), and the radiotherapy dose (0, 2 Gy, 6 Gy or 10 Gy). This analysis showed a significant influence of all studied parameters on the variation of spheroid cell metabolic activity ($p=10^{-6}$ for all parameters) (**Table 2**). More, the cell metabolic activity variance was impacted similarly by the both factors of time Olaparib exposition (37%) and radiotherapy dose (42%).

Secondly, the sensitivity of each spheroid model (MDA-MB-231 or SUM1315) was analyzed by the time, whatever the treatment regimen (+/- Olaparib and +/- radiotherapy) or specifically with 5 μ M Olaparib and fractioned radiotherapy, the treatment condition exhibiting the best efficacy (**Fig. 7**). The ANOVA analysis confirmed the long-term and low dose Olaparib efficacy on both cell lines ($20\pm0.4\%$ and $46\pm0.3\%$ of metabolic activity for MDA-MB-231 and SUM1315 spheroids, respectively, after 5 μ M and 10 Gy radiotherapy) with class effect $<10^{-6}$. Moreover, whatever the treatment condition, MDA-MB-231 spheroids seemed to be more sensitive than SUM1315 spheroids ($45\pm5\%$ and $63\pm2\%$ for MDA-MB-231 and SUM1315 spheroids after 10 days of culture, whatever the treatment condition, with class effect $<10^{-6}$) (**Fig. 7**).

6 The difference in SUM1315 and MDA-MB-231 TNBC cell lines sensitivity is explained by a strong genomic heterogeneity

The ANOVA analysis of both 2D and 3D cell cultures showed a difference in cell survival and spheroid metabolic activity variance explanation of 8% and 5% considering the type of cell line (*i.e.* MDA-MB-231 and SUM1315), respectively (**Table 1 and 2**). Thus, in order to explain this difference, the transcriptome of MDA-MB-231 and SUM1315 cell lines was retrieved from GEODatasets database and analyzed using a classical bioinformatics pipeline including

TOPHAT and DESeq2 package. This analysis underlined 3870 significant differentially expressed genes (DEGs) between the two cell lines. Among these DEGs, 1704 (44%) were overexpressed in MDA-MB-231 cell line whereas 2166 (56%) were overexpressed in SUM1315 cell line (**Fig. 8A**).

Then, we focused on those DEGs involved in the DNA reparation pathways that play a major role in Olaparib and radiotherapy efficacy (**Ollier et al., 2015**)(**Fig 8B**). Among all key selected genes, CHEK1 (log fold change LFG of 2.3, $p < 0.05$), AURKA (LFG = 2.1, $p < 0.01$), LIG1 (LFG = 1.7, $p < 0.01$), RAD51 (LFG = 1.5, $p < 0.01$) and EXO1 (LFG = 2.2, $p < 0.01$) were significantly overexpressed in MDA-MB-231 against SUM1315. Conversely, DNAPK (LFG = 1.88, $p < 0.01$), XLF (LFG = 2.82, $p < 0.01$), MRN (LFG = 5.5, $p < 0.01$), PMS2 (LFG = 2.1, $p < 0.01$) and PARP3 (LFG = 1.3, $p < 0.01$) were overexpressed in SUM1315 cell line compared to MDA-MB-231 cell line (**Fig. 8B**). In contrast, concerning the principal targets of PARP inhibitor Olaparib that are PARP1, and PARP2, no significant difference in gene expression was detected. These results suggest a strong heterogeneity in DNA reparation capacity between these two cell lines (**Fig. 8B**).

Then, for a further insight into the differential phenotypes between the two cell lines, we next used the powerful Ingenuity Pathway Analysis (IPA) software to infer the functional phenotype of each cell line from their differential transcriptomic patterns (**Fig. 8C**). First, for both cell lines, the cell survival pathway was highly activated (z-score > 7). In contrast, an important number of cellular functions were differentially activated between these two cell lines. Indeed, for MDA-MB-231 cell line, the proliferation, migration and invasion capacities were highly activated (z-score > 3 for cellular functions). Conversely, SUM1315 cell line did not exhibit the same cellular pattern of proliferation and migration as MDA-MB-231 but presented a particular phenotype highly associated with neuron development and morphogenesis characteristics (z-score > 2.5). All these results showed highly different cellular phenotypes between the two cell lines.

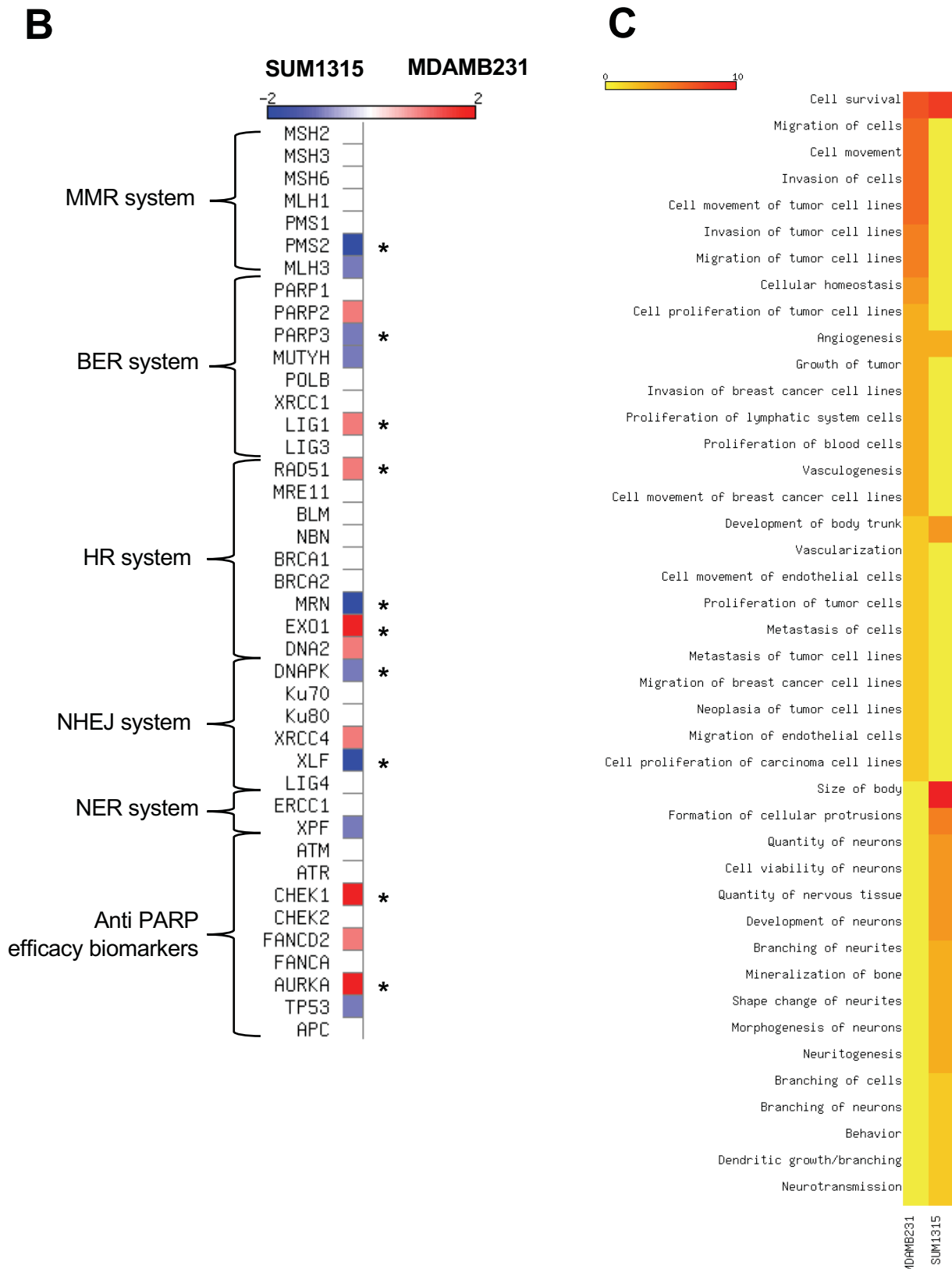


Fig. 8. Transcriptomic analysis of MDA-MB-231 and SUM1315 cell lines: determination of cell difference in sensitivity by a Differential Expressed Gene analysis (A, B) and a functional phenotype analysis (C).

(B) Expression levels of keys genes involved in DNA reparation mechanisms.

The heatmap shows the expression level of key genes involved in different DNA reparation pathways. These last are indicated beside the heatmap. Asterisk marks designated genes which are significantly differentially expressed genes between the two cell lines: MDA-MB-231 and SUM1315.

(C) Determination of MDA-MB-231 and SUM1315 cell lines functional phenotype by an IPA analysis. The heatmap shows the cellular functions that were inferred to be associated with enriched DGE clusters between MDA-MB-231 and SUM1315 cell lines.

DISCUSSION

The Triple-Negative breast cancers (TNBC) are very aggressive and of poor prognostic. This tumor subtype is insensitive to hormone therapy or targeted therapy against HER2 receptor. Actually, the treatment is often composed of surgery, followed by radiotherapy, and conventional chemotherapy, leading to 50% mortality due to escape and resistance mechanisms (**Vinh-Hung and Verschraegen, 2004; Yeo et al., 2014**). Thereby, new treatment strategies such as PARP inhibitors are increasingly developing in order to improve the prognosis of patients. This class of targeted drugs exploit the concept of synthetic lethality (**Dhillon et al., 2016; Fang, 2014; Fang et al., 2011; Farmer et al., 2005; Lord and Ashworth, 2017; O'Neil et al., 2017**). This concept has been defined as a situation in which two gene deficiencies taken separately have no effect, but become lethal when they are combined (**Fang et al., 2011; Farmer et al., 2005; Pernin et al., 2014**). In that case of PARP inhibitors, the deficiency is related to the DNA damage repair systems of Base Excision Repair and Homologous Recombination. The deficiency of both systems leads to accumulation of DNA Double Strand Breaks and ultimately cell apoptosis (**Bryant et al., 2005; Dikomey et al., 1998; Lieber, 2010; Rass et al., 2012; Telli et al., 2018; Wang et al., 2018**). TNBC cancer cells exhibit several DNA repair pathway deficiencies, such as deleterious mutations on the BRCA1/2 genes or assimilated "BRCAness" phenotypes, leading to an increased sensitivity to anti-PARPs.

In addition to being used as monotherapy treatment agents, PARP1 inhibitors can be administered in combination with chemotherapy or radiotherapy. Indeed, the cytotoxicity of radiations is mainly related to the rate of DNA double-strand breaks (**Chalmers et al., 2010; Kötter et al., 2014**). Thus, a Gray of ionizing radiations induces an average number of 1000 CSBs against only 40 DSBs (**Burkart et al., 1999; Ward, 1988**). Thereby, the co-treatment with anti-PARP1 combined to radiotherapy exhibits a strong rational, as both treatments induce apoptosis by the increase of DNA double strand breaks (**Lesueur et al., 2017**). More, inhibition of PARP1 would increase the radio-sensitivity of tumour cells exhibiting a BRCAness phenotype (**Gani et al., 2015; Kötter et al., 2014; Pernin et al., 2014; Senra et al., 2011; Verhagen et al., 2015**).

The development of such treatment optimization strategies has to be conducted in preclinical studies. Since few decades, new techniques mimicking more accurately the tumour microenvironment and kinetics are increasingly developing. These techniques implies the development of three-dimensional cell cultures or "spheroids", *via* several adapted methods

(Antoni et al., 2015; Bissell, 2017; Breslin and O'Driscoll, 2016; Charoen et al., 2014; Lin et al., 2008). 3D cell culture models were developed in our team on several Triple-Negative breast cancer cell lines using the liquid overlay method. More, we demonstrated that according to drug's mechanism of action, i.e. cytotoxic or cytostatic, the choice of a proliferative or non-proliferative 3D model is decisive for drug efficacy's prediction (Dubois et al., 2017). Thereby, the use of spheroids for treatment optimization has been encouraged in several studies, which demonstrated a strong interest of this type of culture to study the toxicity and the resistance of spheroids after radiotherapy and or chemotherapy (Anastasov et al., 2015).

In order to develop new treatment strategies for TNBC tumours, our study aimed to model the effectiveness of a co-treatment anti-PARP (Olaparib) and fractioned radiotherapy. Indeed, the anti-PARP1 Olaparib® recently obtained its New Drug Approval (NDA) for the long-term treatment of metastatic ovary and fallopian tubes cancers exhibiting BRCA1 or 2 mutations and for metastatic breast cancer HER2 non-overexpressed in the US (Deeks, 2015; Dent et al., 2013; Duma et al., 2018; Gladieff et al., 2017; Llombart-Cussac et al., 2015; **Oncology News Burst**). In our experiments, two TNBC cell line models *i.e.* MDA-MB-231 and SUM1315 were cultured in 2D and 3D conditions. The irradiation protocol was carried out in a fractioned regimen of 2 Gy (5 sessions), on a dose and device used for the treatment of breast cancers in clinics, in order to better reflect the transferability of this modeling. Thus, the Olaparib treatment started three hours before the first radiotherapy session and lasted for one day (in 2D) to five days (for 3D) after the last radiotherapy session. This minimum delay of one day before analyzing cell survival allowed the DNA repair mechanisms to be activated. More, this better reflected the cytotoxicity associated with the treatments.

The effectiveness of the cotreatment was firstly determined on 2D cell cultures by the analysis of the number of DNA double-strand breaks with a focigammaH2AX immunomarking. Indeed, the focigammaH2AX histones are the first proteins recruited in the process of DSBs reparation by the HR system (Mah et al., 2010; Mahrhofer et al., 2006; Olive and Banáth, 2004). Their fixation on the lesions sites is directly correlated to the number of DSBs in the nuclei, prior to the initiation of the reparation processes. This marking is usually processed to determine the cells synthetic sensitivity to DNA damaging agents or radiations (Gani et al., 2015; Mah et al., 2010). In our experiments on MDA-MB-231 and SUM1315 cell lines treated with 5 and 50 μ M Olaparib, the number of DNA double strand breaks per nuclei increased in an Olaparib-dose-dependent-manner with 2.1 to 2.3-fold higher than 0.1% DMSO controls, respectively and was systematically higher in presence of the radiotherapy regimen with 2.4 to 3.5-fold higher than the irradiated controls, respectively. These results suggest the synergistic effect of the cotreatment in terms of DSBs generation, probably due to (i) the radiation and (ii) the

accumulation of unrepaired single-strand breaks by the inhibition of the BER repair system, thus degenerating into DSBs.

Then, always in 2D cell culture condition, the cell overall viability and the clonogenic survival of both cell lines were studied after a treatment with increasing concentrations of Olaparib (from 0.1 to 100 μ M for viability test and from 5 to 50 μ M for clonogenic test) and fractionated radiotherapy doses of 2 Gy (from 2 to 10 Gy cumulatives).

For cell viability analysis, Olaparib action on both cell lines was dose and time-dependent. Indeed, after 120h of treatment, the decrease in cell viability in presence of 100 μ M compared to 0.1 μ M was 4.0-fold for MDA-MB-231 and 2.6-fold for SUM1315 cell line. More, in presence of 100 μ M Olaparib, the ratio 24h / 120h of treatment was 3.7 and 2.3-fold for MDA-MB-231 and SUM1315 cell lines, respectively. Similarly, the action of radiotherapy alone on cell viability was fraction-dependent, with a ratio 10 Gy (5 fractions of 2 Gy/day) / 2 Gy (1 fraction) of 1.9 and 1.4-fold viability decrease for MDA-MB-231 and SUM1315 cell line, respectively. Then, in presence of the co-treatment, the overall cell viability was compared to the treatment with Olaparib alone or radiotherapy alone. Interestingly, the co-treatment seemed to be more effective in presence of low doses of Olaparib. Indeed, with 0.1 μ M Olaparib, the ratio of cell viability Ola/Ola+10 Gy was 5.5 and 1.5-fold for MDA-MB-231 and SUM1315 cell line, respectively. In contrast, this ratio was only of 1.3 and 1.0-fold with 100 μ M Olaparib, respectively. These results were confirmed by the ANOVA analysis performed on all dataset, showing a strong interaction of all studied parameters and a strong benefit to propose a co-treatment with low doses of Olaparib. The clonogenic cell survival test is commonly processed to determine the tumor potential of repopulation and though tumor radiosensitivity. Indeed, it evaluates the potential of a cell to form a colony (a cluster of at least 50 contiguous cells) after a treatment. It is usually carried out in two stages, with a first step of cell treatment, and a second step of cell re-seeding with a sufficient incubation time enabling the cells to grow. The colonies are then counted and their number in the radiotherapy or chemotherapy arm is compared to their respective controls (**Munshi et al., 2005**). In these experiments, the clonogenic assay on both cell lines demonstrated the effectiveness of the co-treatment in presence of low doses of Olaparib and radiotherapy 10 Gy. Indeed, with 0.5 μ M Olaparib, the ratio Ola/Ola + 10 Gy was respectively of 100 and 650-fold for MDA-MB-231 and SUM1315 cell lines. In addition, the results from both viability and clonogenic tests highlighted a cell line-dependent effect in response to the co-treatment. Indeed, MDA-MB-231 cell line appeared to be more sensitive than SUM1315.

Then, for a better prediction of the co-treatment effectiveness on more tumor-predictive models, the proliferation, metabolic activity and viability / mortality profile of MDA-MB-231 and SUM1315 spheroids treated at long-term (6 to 10 days) with Olaparib alone (5 and 50 μ M) or concomitantly with fractioned radiotherapy (2, 6 and 10 Gy) were studied. Firstly, for the proliferative MDA-MB-231 spheroid model, Olaparib showed a cytostatic and cytotoxic effect that was dose and time-dependent. In addition, the impact of the co-treatment was again more effective in presence of low doses of Olaparib. Indeed, the decrease in MDA-MB-231 spheroid size after Ola/Ola + 10 Gy was of 1.7-fold with 5 μ M Olaparib and was only of 1.1-fold with 50 μ M. More, the metabolic activity decrease in spheroid was higher in presence of low dose of Olaparib (5 μ M) with a ratio Ola/Ola + 10 Gy of 3.3-fold compared to 0.9 with 50 μ M. In contrast, for the SUM1315 non-proliferative 3D model, Olaparib showed only a dose and time-dependent cytotoxic activity. More, the cotreatment Olaparib / fractioned radiotherapy increased this cytotoxic effect. Indeed, SUM1315 spheroid size decreased slightly, but the spheroid metabolic activity decreased of 1.2-fold with 5 μ M Ola/Ola + 10 Gy against 0.9-fold with 50 μ M. All these experiments demonstrated that the strongest effect detected on all spheroid parameters was in presence of the 5 μ M Olaparib dose combined to 10 Gy radiotherapy. Thereby, these results suggest an important cytotoxic effect on spheroids at long-term and in presence of low doses of Olaparib.

Otherwise, the combination anti-PARP/radiotherapy has been also studied in preclinical studies for the treatment of various types of cancers and has demonstrated improved efficacy compared to anti-PARP treatments alone or radiotherapy alone. Indeed, *in vivo* studies evaluated the impact of Olaparib combined with fractioned radiotherapy sessions (5 x 2 Gy) on bronchial cancer xenografts. These experiments led to delayed tumor growths (**Senra et al., 2011**). Other studies showed similar results on breast cancer xenografts (MCF-7 cell line), where veliparib (another PARP inhibitor) was associated with 6 Gy of hypofractioned X-ray session (**Efimova et al., 2010**). The *in vitro* study of Mao *et al.* (**Mao et al., 2018**) showed a strong cytotoxic effect of the co-treatment on cholangiocarcinoma, a very malignant and radioresistant tumor model, with hypofractioned radiotherapy (0 to 6 Gy) and 10 μ M of anti-PARP1 Olaparib. Interestingly, the cell lines did not carry deleterious variants in BRCA1 or BRCA2 genes but exhibited a *BRCAness* phenotype, leading to a deficient Homologous Recombination system. More, *in vivo* studies on prostate cancer demonstrated significant growth delay and clonogenic kill after hypofractioned or fractioned radiotherapy, coupled to Olaparib (**Gani et al., 2015**).

In our experiments, the sensitivity was heterogeneous between the two cell lines as depicted in the ANOVA analysis. Indeed, in both 2D and 3D cell culture, MDA-MB-231 cell line was

more sensitive to the co-treatment 5 μ M Olaparib / radiotherapy than SUM1315 cell line. These two cell lines are highly invasive and metastatic models from basal B subtype. However, the MDA-MB-231 model was derived from a pleural effusion and exhibits an allelic loss in BRCA1 gene (BRCA1+/-) (De Soto and Deng, 2006; Elstrodt et al., 2006; Neve et al., 2006). In contrast, SUM1315 was derived from a highly invasive infiltrated ductal skin metastasis (Barnabas and Cohen, 2013; De Soto and Deng, 2006; Elstrodt et al., 2006; Fillmore and Kuperwasser, 2008; Prat et al., 2010) that carries deleterious mutation in the BRCA1 gene (185del AG) leading to a truncated protein (BRCA-/-). Therefore, these cell lines should be sensitive to anti-PARP1 and radiotherapy treatment, as they exhibit a BRCAness status leading to an impaired HR reparation system (Lord and Ashworth, 2016; Turner et al., 2004; Wang et al., 2018).

Thereby, in order to explain the difference in cell sensitivity between the two cell lines, a *in silico* transcriptomic comparison was carried out from GEOdatasets (Boire et al., 2017; Daemen et al., 2013; Marcotte et al., 2016). Firstly, the analysis of the Differentially Expressed Genes (DEGs) in the transcriptome of the two cell lines showed a homogeneous DEG repartition. Secondly, the expression level of key DNA repair systems genes (involved in MMR, BER, HR and NHEJ systems) and associated biomarkers of PARPi efficacy was carried out and compared for the two cell lines. The latter showed no difference in the level expression of PARP-1 and PARP-2 Olaparib targets. In contrast, in MDA-MB-231 cell line, the overexpression of AURKA was detected. Thus, this gene, exhibiting a function on cell cycle regulation, is a biomarker of PARP inhibitors sensitivity (Ihnen et al., 2013; Ohmoto and Yachida, 2017; Telli et al., 2018; Wang et al., 2018). Therefore, this specific overexpression of AURKA could partially explain the increased sensitivity to the co-treatment of MDA-MB-231 model compared to SUM1315 model. Thirdly, in order to highlight the differential functional phenotypes of MDA-MB-231 and SUM1315 cell lines, an Ingenuity Pathway Analysis (IPA) was conducted according to their differential transcriptomic patterns. This analysis highlighted two very different biological phenotypes. Indeed, the MDA-MB-231 cell line exhibited strong proliferation, migration and invasion functions, already reported in previous studies (Dubois et al., 2017). In contrast, SUM1315 cell line expressed a stem-cell like phenotype, with a specific neuronal engagement profile. This general pattern could partially explain the resistant property of SUM1315 cell line, as undifferentiated stem cells exhibit slowed proliferation rates and develop specific resistance mechanisms (Fillmore and Kuperwasser, 2008). Otherwise, the neuronal differentiation pattern in SUM1315 cell line might be explained by its highly metastatic origin (Barnabas and Cohen, 2013). Indeed, triple-negative breast tumors frequently develop metastases in brain, compared to other types that invade preferentially bones or lungs (Rakha et al., 2009).

All these experiments showed the interest of the combinatory regimen including Olaparib and fractioned radiotherapy on the decrease in clonogenic viability, overall viability in 2D, and on the long-term growth inhibition and toxicity in 3D for Triple-Negative breast cancer cell lines. More, these works led to the promising perspective of a (i) daily (ii) low dose and (iii) long-term Olaparib administration coupled to fractioned radiotherapy on resistant metastatic breast cancers, and possibly for brain localizations, given the very difficult handling of these advanced stages (**Franchino et al., 2018; Orecchia, 2018; Takahashi and Isogawa, 2018**).

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AUTHOR CONTRIBUTIONS

C.D., Fa.M., C.H., Fl.M, P.D., C.A., S.G., D.D., L.M., E.M., F.P.L., and M.B. conceived and designed the study.

C.D., Fa.M. and Fl.M. performed the experimental work.

C.H. and S.G. performed the transcriptomic analysis.

C.D., Fa.M, C.H., P.D., E.M., and M.B. analyzed the data.

C.D., Fa.M., F.K. and C.H. performed the statistical analysis.

C.D., Fa.M., Fl.M., C.H. and M.B. wrote the manuscript.

All authors reviewed the manuscript.

ADDITIONAL INFORMATION

Competing interests: The authors declare no competing financial interests.

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

III) Conclusion sur les travaux

Dans un contexte de modélisation *in vitro*, ces travaux de recherche préclinique ont permis de confirmer l'efficacité d'un traitement combinant l'inhibiteur de PARP1 **Olaparib** et la **radiothérapie fractionnée**, sur des modèles des sphéroïdes issus des lignées cellulaires du cancer du sein triple-négatif, en présence de faibles doses d'Olaparib. Cette méthode de culture a également permis d'effectuer des traitements sur de longues périodes et ainsi de modéliser l'efficacité d'un traitement au long-cours avec l'anti-PARP notamment pendant les séances de radiothérapie et plusieurs jours après. Parallèlement, la caractérisation transcriptomique *in silico* des lignées cellulaires MDA-MB-231 et SUM1315 a mis en évidence leur divergence. Cette hétérogénéité génétique pourrait expliquer, chez ces modèles de tumeurs particulièrement agressives et hautement métastatiques, leur mode de réponse à ce co-traitement.

L'effet potentialisateur de l'Olaparib sur la radiothérapie a été retrouvé en présence de faibles doses de la molécule chimiothérapeutique. Afin de caractériser plus finement ce phénomène, des analyses de la pénétration/distribution de l'Olaparib ainsi que l'expression des protéines de chimiorésistance (protéines MDR) ont été effectuées sur ces modèles de culture en trois dimensions, traités par l'Olaparib.

D) Développement de l'Olaparib-FL, un outil de diagnostic pour les modélisations du mode d'action des anti-PARPs en culture 3D

I) Introduction

La modélisation préclinique de l'action des molécules chimiothérapeutiques sur des lignées cellulaires tumorales en conditions 2D et 3D permet de mieux anticiper leur efficacité et leur action prédictive. Ainsi, au cours de notre étude afin de mieux comprendre l'action de l'Olaparib dans les modèles de lignées cellulaires en 2D et 3D en termes de pénétration/distribution et de résistance, un dérivé fluorescent de l'Olaparib-fluorescent intitulé « Ola-FL » a été développé comme outils de diagnostic. Ces outils permettent notamment une analyse dynamique et/ou en « end-point » par microscopie à fluorescence sur des explants tumoraux (en phase de diagnostic ou pour le suivi du traitement), à partir de modèles animaux (**Carney et al. 2018**) ou encore de lignées cellulaires (**Thurber et al. 2013**). Ainsi, le troisième axe de ces travaux de thèse visait à synthétiser et caractériser le dérivé de l'Olaparib fluorescent. Ensuite, la pénétration et la distribution de l'Ola-FL ont été étudiées sur des cultures en monocouches et tridimensionnelles de trois lignées cellulaires du cancer du sein triple négatif MDA-MB-231, SUM1315 et HCC1937. **Ces travaux ont fait l'objet d'une publication en premier auteur.**

II) (Article rédigé) Olaparib-FL development for the evaluation of anti-PARP penetration and distribution on 3D cell cultures of triple-negative breast cancer models.

Olaparib-FL development for the evaluation of anti-PARP penetration and distribution on 3D cell cultures of triple-negative breast cancer models.

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ABSTRACT

Triple-Negative breast cancers (TNBC) are very aggressive and of poor prognostic. Poly-ADP-ribose Polymerases inhibitors (PARPi) are a class of synthetic lethality drugs targeting tumours exhibiting “BRCAness” phenotypes, such as TNBC. In these works, for the modelization of the anti-PARP1 Olaparib efficiency on TNBC cells lines, an Olaparib fluorescent derivative (Olaparib-FL or “Ola-FL”) was firstly synthesized and characterized. Then, the penetration and distribution of Ola-FL was determined on MDA-MB-231, SUM1315 and HCC1937 TNBC cell lines, cultured in monolayer of three-dimensional conditions. The results showed first, Ola-FL production yield was 65% with a purity higher than 99%. Then, Ola-FL penetration analysis in three TN cell line models in monolayer culture showed a rapid penetration of this anti-PAPRP in cells and its accumulation in nucleus. The same study on 3-day-old spheroids showed a rapid penetration in 5 min and a homogenous Ola-FL repartition and localization in the nuclei throughout the integrality of the three spheroid models. Similarly, same Ola-FL distribution and localization was detected on spheroids cultured for 14 days, demonstrating the fixation of this molecule even in hypoxic spheroid areas. All these results demonstrated the suitability of this Olaparib fluorescent derivative to model the distribution / efficacy of anti-PARP in order to optimize the predictive treatment response in TN tumors.

KEYWORDS

PARP inhibitors, fluorescent Olaparib, Triple-Negative breast cancer, 3D cell culture

INTRODUCTION

The very aggressive Triple-Negative breast cancers (TNBC) are insensitive to hormone therapy or targeted therapy against HER2 receptor (Trastuzumab®) (**Foulkes et al., 2010**). For this subtype, the treatment is mainly composed of surgery, followed by radiotherapy and conventional chemotherapy (**Anders and Carey, 2009; Penault-Llorca and Viale, 2012**). These strategies are effective on approximately 50% of patients exhibiting a relatively high 5-years overall survival. Unfortunately for the other half of patients with TNBC, a rapid and aggressive recurrence by the development of metastases is encountered, leading to very bad outcomes (**Duma et al., 2018; Lehmann et al., 2016**). Nevertheless, new treatment strategies were developed in order to improve the prognosis of patients, that are PARP inhibitors. This class of targeted drugs exploit the concept of synthetic lethality (**Fang et al., 2011; Farmer et al., 2005**), which has been defined as a situation in which two deficiencies which, taken in isolation, have no effect, but become lethal when they are combined (**Fang et al., 2011; Farmer et al., 2005; Pernin et al., 2014**). In that case of PARP inhibitors, the deficiency is related to the DNA damage repair systems. Indeed, the Homologous Repair system (HR), mediated by the BRCA1 and 2 proteins, is the one that allows the most faithful repair of DSBs, thus preserving genomic integrity. However, a great proportion of TNBC tumours exhibit deleterious mutations on the BRCA1/2 genes or assimilated "BRCAness" phenotypes (**Telli et al., 2018; Wang et al., 2018**) leading to deficient HR mechanism in these cells. Thus, PARP inhibitors would inhibit the action of the nuclear enzyme PARP1 which plays (i) a dominant role in repairing single-strand breaks (Base excision Repair system BER) and (ii) in the alternative non-faithful repair pathway for double-stranded DNA breaks by non-homologous extremal ligation (NHEJ). Therefore, treatment of TNBC tumours with a PARP inhibitor would lead to the accumulation of single-strand breaks (SSB) that would be converted into DSBs during the passage of replication forks. The latter would no longer be repaired by the HR system deficient because of the "BRCAness" status of TNBC tumours leading to the death of tumor cells by apoptosis (**Bryant et al., 2005**).

Among all PARP inhibitors developed in preclinical and clinical studies, the Olaparib® recently obtained its New Drug Approval (NDA) for the treatment of ovary and fallopian tubes metastatic cancers BRCA1 or 2 mutations carriers, in the second treatment line after failure of conventional chemotherapies based on platinum salts (**Deeks, 2015; Dent et al., 2013; Gladiëff et al., 2017; Llombart-Cussac et al., 2015**). Nevertheless, the analysis of anti-PARPs efficiency and action mechanisms is still necessary for the improvement of the treatment strategies. Therefore, some fluorescent derivatives also called "PARPi-FL" were

developed in order to determine the distribution and to monitor the pharmacokinetics of PARP inhibitors in *in vitro* and *in vivo* models, such as glioblastoma, head and neck cancers, breast or ovarian cancers (Irwin et al., 2014; Kossatz et al., 2016; Thurber et al., 2013, 2014). These PARPi-FL are also developed as companion diagnostics enabling patient selection, treatment planning and monitoring as well as intraoperative imaging (Kossatz et al., 2018).

The *in vitro* experiments are mainly realised in monolayer cell culture. Nevertheless, preclinical experiments are increasingly performed in more biomimetic models that are the 3D cell cultures. These models also called “spheroids”, mimic more precisely the tumour microenvironment, by the formation of genetical, biochemical and phenotypical gradients. Therefore, the 3D cell culture an adapted tool for drug screening and evaluation (Antoni et al., 2015; Bissell, 2017; Breslin and O’Driscoll, 2016; Charoen et al., 2014)(Costa et al., 2016; Dubois et al., 2017; Fang and Eglen, 2017). Our team already developed several Triple-Negative breast cancer 3D models. These works specifically highlighted the importance of determining the proliferative / non-proliferative property of the 3D models, in order to proceed to optimized 3D drug screenings (Dubois et al., 2017).

To model the effectiveness of anti-PARP on TNBC cells lines, these works aimed on the synthesis and characterisation of an Olaparib fluorescent derivative (Olaparib-FL or “Ola-FL”) and the analysis of its penetration and distribution in three TNBC cell lines models, *ie* MDA-MB-231, SUM1315 and HCC1937, in 2D and 3D cell culture conditions.

MATERIAL AND METHODS

1. Olaparib-FL synthesis and characterization

1.1. Synthesis

Ola-FL was synthesised by coupling 4-[[4-fluoro-3-(piperazine-1-carbonyl)phenyl]methyl]-2*H*-phthalazin-1-one, a synthetic Olaparib analog synthesized as previously described (Menear et al., 2008), to the commercial BODIPY-FL fluorescent probe (Interchim, Montluçon, France). A solution of BODIPY-FL succinimidyl ester (15.0 mg, 38.5 µmol) in acetonitrile (600 µl) was added to a solution of 4-[[4-fluoro-3-(piperazine-1-carbonyl)phenyl]methyl]-2*H*-phthalazin-1-one (14.1 mg, 38.5 µmol) and triethylamine (27 µl, 193 µmol) in acetonitrile (600 µl). The reaction mixture was stirred for 24 hours at room temperature and concentrated under vacuum.

The residue was purified by column chromatography (SiO₂, 40-63 μm, Dichloromethane/Methanol, 95/5) to yield Ola-FL as a red solid (16.0 mg, 25.0 μmol, 65%).

1.2. Purity

The purity of Ola-FL was assessed by analytical HPLC-UV/Vis-DAD analysis using a HP 1100 series LC system equipped with an online degasser, a quaternary pump, a column oven, a photodiode array detector (DAD) and an autosampler. Water and acetonitrile (ACN) were used for the preparation of the mobile phase. The temperature of the column oven was kept at 25 °C. A gradient elution was applied at a flow rate of 1.0 mL/min through a reversed-phase Nova-Pak® 4 μm C18 column (150 x 3.9 mm; Waters Corporation, Milford, MA, USA) with an additional Nova-Pak® guard column. The following gradient was employed: 0 – 15 min, 10 – 90% ACN; 15 – 17 min, 90 – 10% ACN. A sample of Ola-FL in ACN with a volume of 10 μL was injected. UV spectra were recorded from 220 to 650 nm from 0 to 16 min and the monitoring wavelength for Ola-FL were set to 254 and 506 nm. Agilent ChemStation software (version B.04.03-SP1, Agilent Technologies, France) was used for acquisition of chromatograms, spectra and integration data.

1.3. Characterization

Ola-FL was fully characterized by ¹H nuclear magnetic resonance (¹H NMR), electrospray ionization mass spectrometry (MS-ESI), high-resolution electrospray ionization mass spectrometry (HRMS-ESI) and fluorescence spectroscopy. NMR spectra were recorded on a Bruker AVANCE 400 (400 MHz) spectrometer. Chemical shifts for protons are reported in parts per million (ppm) and are referenced against the dimethylsulfoxide lock signal (¹H, 2.50 ppm). Data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quadruplet, m = multiplet), coupling constants (Hz), and integration. Mass spectra were recorded on a high-resolution Waters Micro Q-ToF apparatus in positive mode (UCA-Partner, Université Clermont Auvergne, France). Absorption and emission spectra were recorded on the Cytation3MV.

¹H NMR (400 MHz, DMSO-*d*₆) δ = 12.60 (s, 1H), 8.27 (d, *J* = 7.8, 1H), 7.97 (d, *J* = 7.6, 1H), 7.90 (t, *J* = 7.6, 1H), 7.83 (q, *J* = 7.7, 1H), 7.70 (s, 1H), 7.44 (m, 1H), 7.38 (m, 1H), 7.24 (t, *J* = 9.0, 1H), 7.10 (s, 1H), 6.42 (dd, *J* = 13, *J* = 4.1, 1H), 6.31 (s, 1H), 4.34 (s, 2H), 3.70-3.50 (m, 4H), 3.41 (m, 2H), 3.18 (m, 2H), 3.09 (m, 2H), 2.75 (td, *J* = 27, *J* = 7.9, 2H), 2.47 (d, *J* = 5.3, 3H), 2.27 (s, 3H). **MS-ESI** (+) *m/z* = 621.3 [M – F]⁺, 641.3 [M + H]⁺, 663.2 [M + Na]⁺. **HRMS-ESI** [M + H]⁺ *m/z* calculated for [C₃₄H₃₃O₃N₆BF₃]⁺ 641.2657, found 641.2664. **Absorption and emission maxima** were determined at 506 and 520 nm, respectively.

2. Cell culture

2.1. 2D cell culture

The three triple-negative breast cancer **SUM1315** (MO2TM, Asterand), **MDA-MB-231** (HTB-26TM, ATCC®) and **HCC1937** (CRL2336TM, ATCC) cell lines were cultured either in Ham's F12 medium (Gibco®) or RPMI 1640 medium (GIBCO®). For the culture of **SUM1315** cell line, the Ham's F12 was supplemented with 5% decompemented fetal calf serum, 10 mM HEPES buffer (Sigma®), 20 µg/mL gentamycin (Panpharma®), 10 ng/mL EGF (Sigma®) and 4 µg/mL insulin (NovoRapid®). For **MDA-MB-231** and **HCC1937** cell lines cultures, the RPMI1640 medium was supplemented with 10% decompemented fetal calf serum and 20 µg/mL gentamycin. The cells were seeded at a concentration of 10 000 cells/cm² in 75 cm² flat culture flasks (Falcon®) containing 15 mL of medium. At 90% confluence, cells were detached with trypsin, counted with a blue trypan exclusion test and re-seeded in new culture flasks at the same concentration. For all experiments, cell cultures were maintained in a humidified incubator set at 37°C and 5% CO₂. The cell lines were not experimented after passage 45 and the absence of mycoplasma contamination was systematically checked before running the cell experiments (Mycoplasma Check, GATC®).

2.2. 3D cell culture

For spheroid formation with three TNBC cell lines (**MDA-MB-231**, **SUM1315** and **HCC1937**), the liquid overlay technique was performed according to previous published works of the team (**Dubois et al., 2017**).

3. Cell treatment

Ola-FL was dissolved in DMSO. 2D and 3D cell cultures were treated with 1 µM Ola-FL. The final concentration of DMSO was of 0.1% in all cell cultures. For monolayer experiments with Ola-FL, cells were seeded at a concentration of 50 000 cells/well in Ibitreat chamber slides (Ibidi®) and let to attach for 24h in the incubator. For 3D cell culture experiments, spheroids (1000 cells/well) were treated after 3 or 14 days of culture.

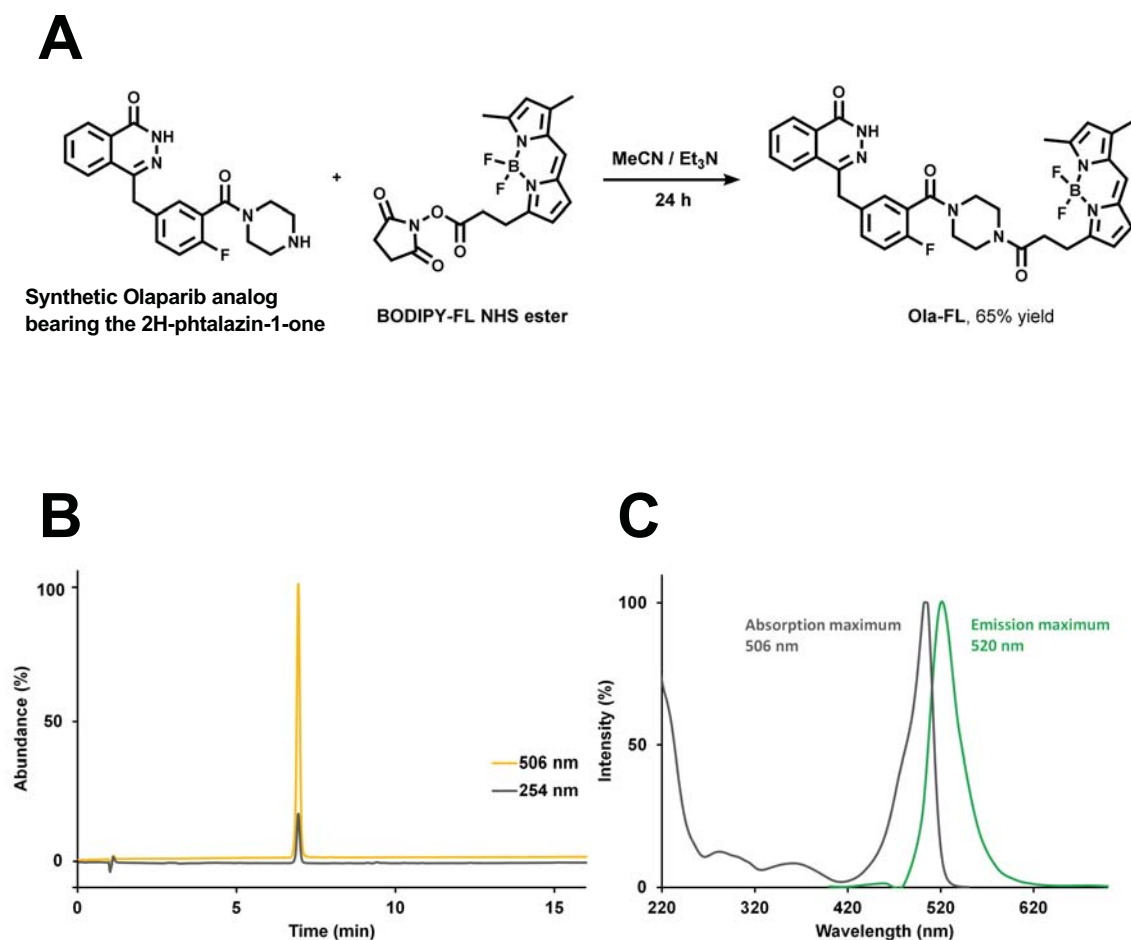


Figure 1:

Olaparib-FL synthesis and characterization (A) Synthesis of Ola-FL, a fluorescent PARP inhibitor. Ola-FL was synthesized by coupling a synthetic Olaparib analog to the commercial BODIPY-FL NHS ester fluorescent probe. Ola-FL characterization, (B) Representatives HPLC traces at 254 and 506 nm (same scale) of Ola-FL after purification, (C) Absorption and emission spectra of Ola-FL.

4. Cell culture imaging with Cytation3MV

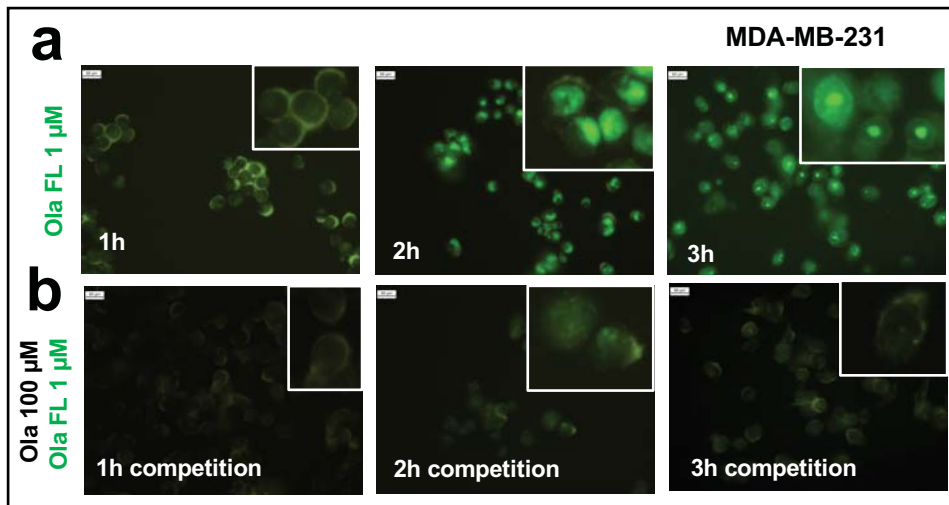
For the analysis of Ola-FL localisation and distribution, the three cell lines were incubated with Ola-FL for 1h, 2h or 3h. After each incubation time, cells were washed twice in cold PBS and directly observed with the Cytation3MV (DAPI and GFP filters). For competition tests with Olaparib, cells were firstly incubated with 100 μ M Olaparib for 1h, washed three times in PBS and finally incubated with Ola-FL for 1h, 2h or 3h. Both localisation and competition tests were imaged and analysed with the Cytation3®MV multimode plate reader (Biotek).

Spheroids obtained from the three cell lines were incubated with Ola-FL for 5 min, 30 min or 1h (for spheroids of 3 days culture) or for 3h (for spheroids of 14 days culture). After each incubation time, spheroids were washed three times in cold PBS and fixed in 4% paraformaldehyde (PAF 4%). The water was progressively removed from the samples with successive incubations in 15% and 30% sucrose. Then, the spheroids were coated in a cryomounting medium (OTC Tissue Freezing Medium, Leica), quickly freezed in liquid nitrogen and cut into 10 μ m slides with a cryostat (CICS, Clermont-Ferrand). After 24h drying, the slides were stained with 10 μ /mL Propidium Iodide or Hoechst and then mounted with ProlongDiamond antifade mountant (ThermoFisher).

RESULTS

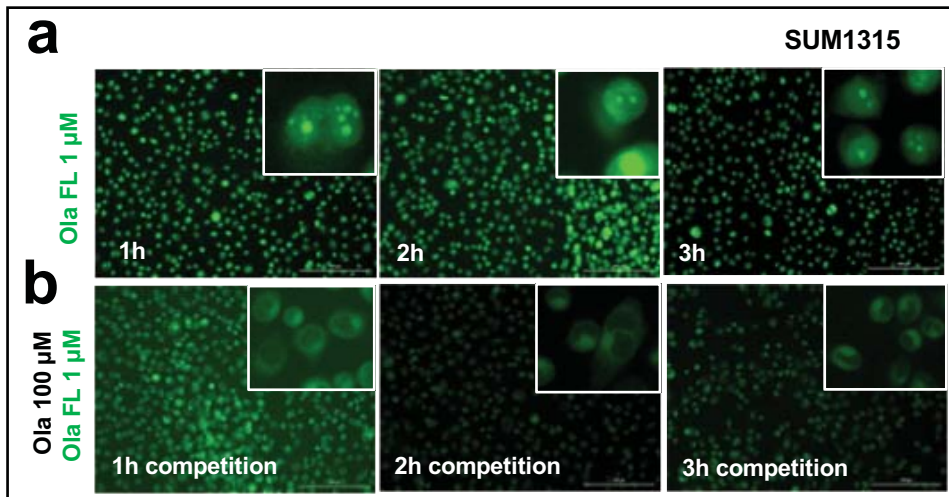
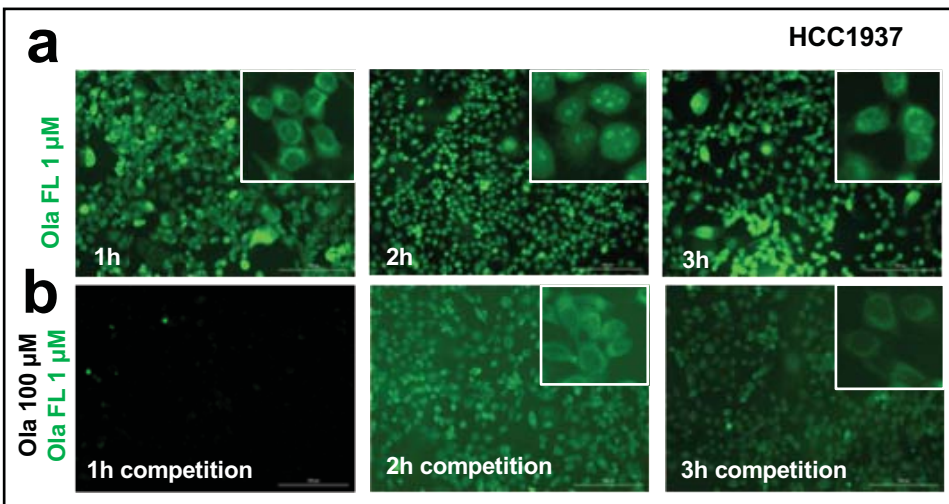
1. Olaparib-FL

Ola-FL is a fluorescent agent which exhibits structural similarity and behavior *akin* the PARP inhibitor Olaparib. As described in **Fig. 1A**, Ola-FL was synthesised by coupling a synthetic Olaparib analog bearing the 2*H*-phtalazin-1-one active moiety to the commercial BODIPY-FL fluorescent probe. The NHS-ester coupling reaction led to Ola-FL in a 65% yield after a 24-hour reaction time in acetonitrile and purification by liquid chromatography. Ola-FL was then fully characterized by proton nuclear magnetic resonance, high resolution mass spectrometry and fluorescence spectroscopy. Excellent purity (>99%) was established by analytical HPLC-UV-DAD analysis as one single peak was observed on chromatograms on the 220 – 550 nm range. Representatives HPLC traces at 254 and 506 nm (same scale) of Ola-FL after purification are shown on **Fig. 1B**. Absorption and emission spectra of Ola-FL were also recorded, with absorption and emission maxima determined at 506 and 520 nm, respectively (**Fig. 1C**).

A**Figure 2:**

Olaparib-FL penetration and competition test with Olaparib in MDA-MB-231 (A), SUM1315 (B) and HCC1937 (C).

Cells were incubated with Ola-FL 1 μ M for 1h, 2, and 3h, washed and directly imaged with Cytation3MV. For competition tests, prior to Ola-FL incubation, cells were treated with Olaparib in excess (100 μ M) for 1h.

B**C**

2. Olaparib-FL analysis in monolayer cell culture

The penetration of Ola-FL in three TNBC cell lines **MDA-MB-231**, **SUM1315** and **HCC1937** without or with Olaparib competition (100 μ M, in excess) was analyzed after 1h, 2h or 3h of incubation, using the multimode imager Cytation3MV (Biotek) (**Fig. 2**). For the competition experiments, cells were first treated with Olaparib in excess prior to Ola-FL incubation. In MDA-MB-231 cell line, Ola-FL alone was first detected in the cytoplasm of cells after 1h incubation (**Fig. 2Aa**). Then, it penetrated the nuclei after 2h and remained concentrated in one intense “hot spot” after 3h. In contrast, in presence of Olaparib in excess, the overall Ola-FL fluorescent signal remained cytoplasmic and dramatically low during the all experiment (**Fig. 2Ab**). For SUM1315 cell line, Ola-FL alone penetrated into the nucleus after only 1h incubation and remained until 3h. More, the same intense nuclei markings were detected for this cell line at the three incubations times (**Fig. 2Ba**). In presence of Olaparib in excess, Ola-FL signal remained high but concentrated in the cytoplasm after 1h and decreased clearly after 2h and 3h (**Fig. 2Bb**). For HCC1937 cell line, Ola-FL alone was detected in the cytoplasm of the cells after 1h. Then, intense hot spots were present in the nuclei after 2h, that were more diffused after 3h (**Fig. 2Ca**). With 100 μ M Olaparib, no Ola-FL signal was detected after 1h incubation (**Fig. 2Cb**). Nevertheless, after 2h and 3h of competition, Ola-FL signal increased clearly, but remained localized in the cytoplasm (**Fig. 2Cb**). These results firstly showed that for whatever TNBC studied model, Ola-FL rapidly penetrated the cells and reached the nuclei after 1h to 3h. Moreover, a combinatory administration Olaparib / Ola-FL demonstrated clearly a competition for the fixation to their nuclear molecular target.

3. Olaparib-FL distribution in triple-negative breast cancer spheroids

Ola-FL penetration and cell distribution in TNBC spheroids by the time was then studied. For this, the spheroids obtained from **MDA-MB-231**, **SUM1315** and **HC1937** TNBC cell lines aged of 3 (**Fig. 3**) or 14 days (**Fig. 4**), were incubated with 1 μ M Ola-FL for 5 min, 30 min, 1h or 3h. Spheroids cuts and nuclear markings (propidium iodide, red fluorescence) were then processed and the penetration and cell distribution of Ola-FL was studied according to spheroid age and the time of incubation.

First, the analysis of Ola-FL distribution was performed in small spheroids presenting area 8000, 3500 and 5000 μ m² for MDA-MB-231, SUM1315 and HCC1937 spheroids, respectively (**Fig. 3**). For the three cell lines, a rapid penetration and a homogenous distribution of Ola-FL

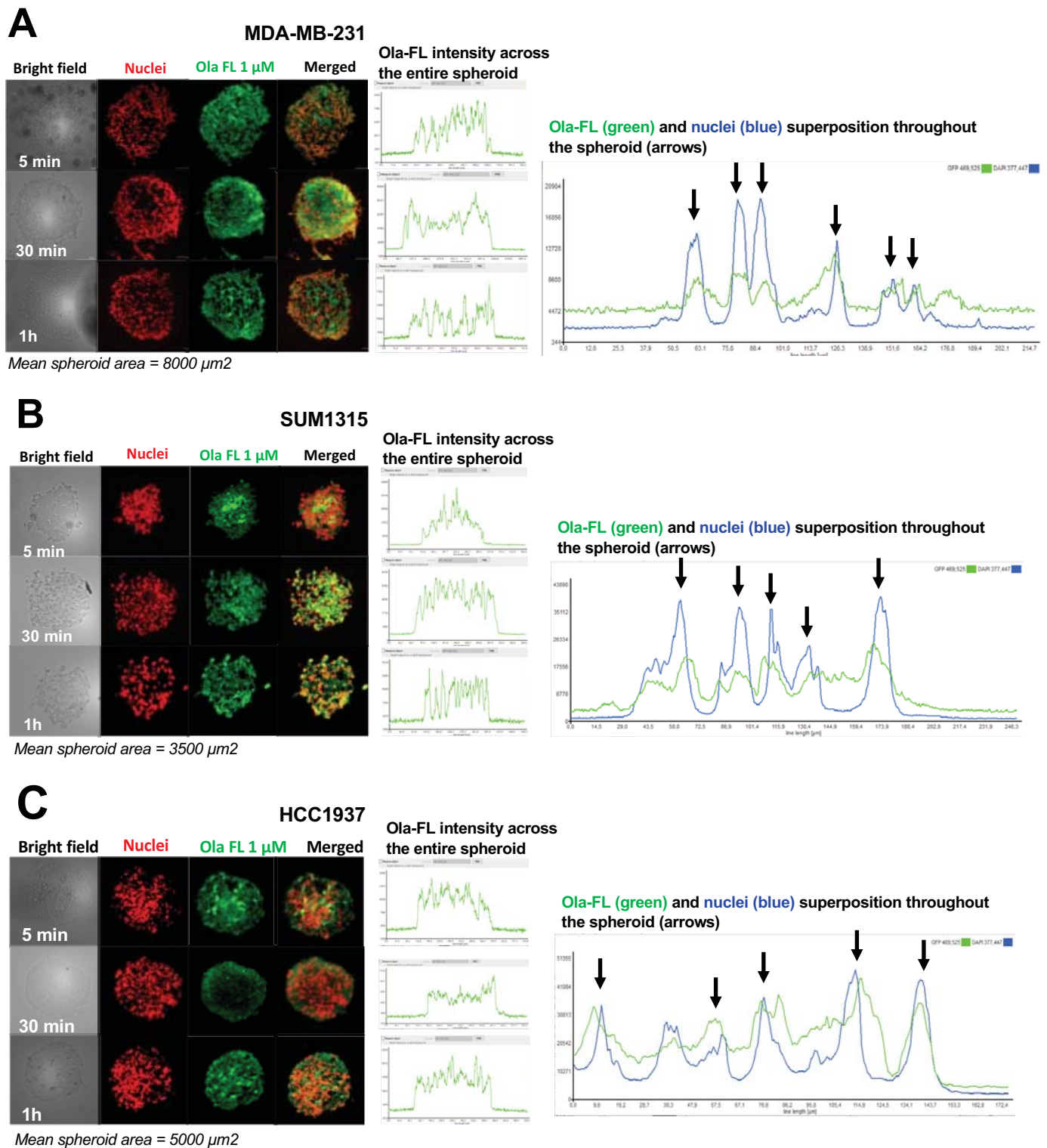


Figure 3: Olaparib-FL penetration and distribution in MDA-MB-231 (**A**), SUM1315 (**B**) and HCC1937 (**C**) spheroids after 3 days of culture. Spheroids were incubated with 1 μ M Ola-FL for 5 min, 30 min and 1h. After each incubation time, spheroids were washed, fixed in PAF 4% and dehydrated in sucrose 15% and 30%. Spheroids were then cut into 10 μ m with a cryostat and stained with Hoescht. Images were acquired with Cytation3MV (Biotek). The repartition and intensity of each fluorescent marker was analysed by the realisation of profile line histograms. **Red marking = nuclei, green marking Ola-FL**

was detected throughout the spheroids after 5 min incubation, as depicted in the green histograms of fluorescence intensity (**Fig. 3A, B, C**). Then, after 30 min and 1h of Ola-FL treatment, the distribution in spheroids remained homogenous. In addition, for the three cell line models cultured in 3D, Ola-FL was localized in the nucleus of the cells, as confirmed with the co-localization histogram (superposition of nucleus marking and Ola-FL marking) (**Fig. 3A, B, C**).

Then, the analysis of Ola-FL distribution was performed in larger spheroids aged of 14 days (**Fig. 4**), presenting area of 25000, 22000 and 20000 μm^2 for MDA-MB-231, SUM1315 and HCC1937 spheroids, respectively. First, the spheroid nuclear marking showed a more intense red marking in the periphery of the three spheroids models obtained from **MDA-MB-231 (Fig. 4A)**, **SUM1315 (Fig. 4B)** and **HCC1937 cell lines (Fig. 4C)**. Parallely, after 3h treatment, Ola-FL remained in the spheroids and its distribution was homogeneous in the three models (green histograms depicting Ola-FL intensity across the entire spheroid).

DISCUSSION

Despite the advances in terms of detection and the development of individualized treatment strategies, breast cancer remains a major public health concern (**Bray et al., 2013; Ferlay et al., 2015**). Thus, among the breast cancer subtypes, the Triple-Negative phenotype frequently mutated for BRCA1/2 genes, is of very poor prognosis (**Anders and Carey, 2009**). For the improvement of patient outcome, small inhibitors of Poly-ADP-ribose polymerases were developed as targeted therapies for BRCA1/2-deficient or associated “BRCAness” tumours (**Bryant et al., 2005**). Among them, the Olaparib, an anti-PARP1, was approved for the treatment of ovarian cancers and is in development for the treatment of breast, head and neck or prostate cancers (**Deeks, 2015**). Though promising results, the action mechanism of Olaparib and other PARPi at the cellular level has still to be investigated. For this, imaging strategies combining the drugs with fluorescent probes are increasingly developed, for preclinical and *ex vivo* clinical analysis (**Irwin et al., 2014**). In this context, these experiments aimed to the development of the PARPi fluorescent derivative Ola-FL for penetration and distribution analysis in 2D and 3D TNBC cell line models. The results showed a rapid penetration (inferior to 3h) of Ola-FL in MDA-MB-231, SUM1315 and HCC1937 cell lines cultured in monolayer. More, the fluorescent drug reached its molecular target (the nucleus) as confirmed in the localization and competition tests. Indeed, the competition test consisted in treating cells with high concentration of Olaparib (100 μM) prior to treat with Ola-FL (1 μM) for 1h, 2h or 3h. If both molecules present the same molecular target, the one deposited in

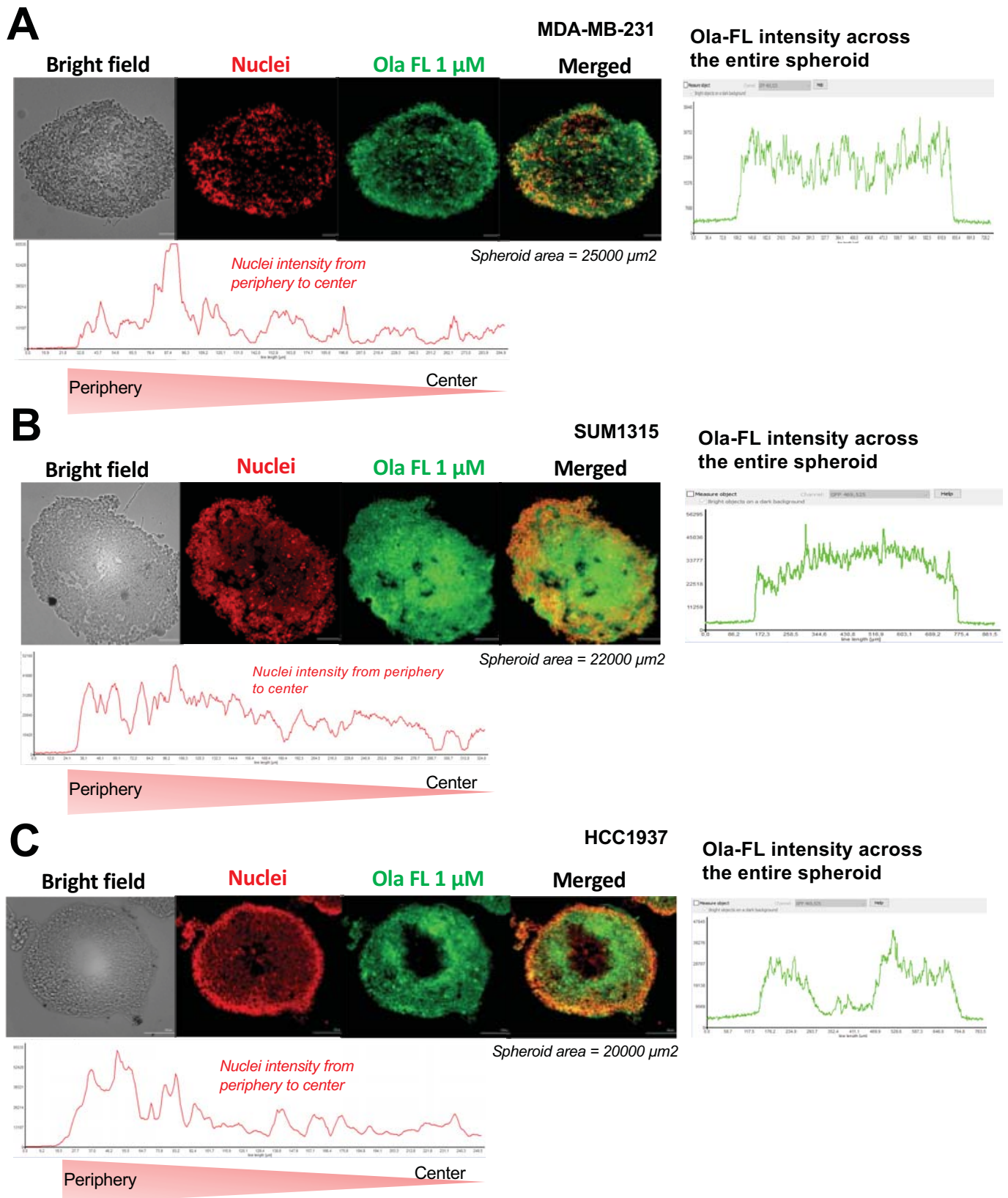


Figure 4: Olaparib-FL distribution after 3h treatment in MDA-MB-231 (**A**), SUM1315 (**B**) and HCC1937 (**C**) spheroids after 14 days of culture. Spheroids were incubated with 1 μM Ola-FL for 3h and then washed three times in PBS. Spheroid cuts were stained with Propidium Iodide and observed with Cytation3MV. **Red marking = nuclei, green marking Ola-FL.** The repartition and intensity of each fluorescent marker was analysed by the realisation of profile line histograms.

excess will prevent the second one to reach to/fix its target. In that case of Ola-FL, this low fixation/access to the molecular site would result in the absence / low fluorescent signal in the nucleus of competed cells (**Carney et al., 2018; Reiner et al., 2012**).

Then, the analysis of Ola-FL penetration and distribution was studied in the three spheroid models after 3 days or 14 days of culture. The choice of these two spheroid culture times was motivated by the will of modeling drug mechanism in several tumour conformations. Indeed, old spheroids present a necrotic / quiescent hypoxic core that might influence molecule penetration, distribution and metabolism (**Costa et al., 2017; Lin et al., 2008**). Thus, our 3D models of 14 days presented decreased propidium iodide markings in the cell nuclei localized in the center. This confirmed the formation of quiescent / senescent cells in the core of our models. More, HCC1937 spheroids aged of 14 days presented a central hole. The latter may be mimicking the acinus-like structure of mammary gland, resembling a normal-tissue-like organization (**Barcellos-Hoff et al., 1989; Furuta and Bissell, 2016**). This can be explained by the differentiated nature of this cell line, issued from a primary triple-negative breast tumour (**Tassone et al., 2003**). In contrast, MDA-MB-231 and SUM1315 cell lines are highly undifferentiated cell lines derived from metastatic breast cancer tumours, that did not keep their capacity of organizing in acinus-like structures (**Barnabas and Cohen, 2013; Neve et al., 2006**).

Therefore, the analysis of Ola-FL penetration in spheroids after 3 days of culture showed a rapid (5 min) and homogenous penetration of the fluorescent compound throughout the integrality of the micro-tumours and this for the three models. Thus, the Ola-FL was localized in the nucleus of the cells. Interestingly, the spheroids of 14 days produced from the three models exhibited the same homogenous repartition than with younger spheroids and this after 3h treatment. Similarly, the Ola-FL was localized in the nucleus of the spheroid cells.

All these preliminary results showed the possibility of using Ola-FL for the analysis of drug mechanism of action in TNBC models. Ola-FL can be used to define the action mode of PARPi in several cancer types, as well as the study of drug penetration and distribution in TNBC models. In our models, the future works will aim to study Ola-FL distribution at long-term (several days of treatment) and to analyze the co-expression of several multidrug resistance proteins, responsible for Olaparib resistance in breast cancer treatment.

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AUTHOR CONTRIBUTIONS

C.D., P.D., C.A., E.M., F.P.L., M.B. conceived and designed the study;

C.D., P.D. performed the experimental work;

C.D., M.B., E.M. analyzed the data;

C.D., P.D., M.B. wrote the manuscript. All authors reviewed the manuscript.

ADDITIONAL INFORMATION

Competing interests: The authors declare no competing financial interests.

III) Conclusion sur les travaux

Ces travaux de développement ont permis de synthétiser avec succès le dérivé fluorescent de l'Olaparib « Ola-FL ». Les expériences de marquage en microscopie à fluorescence sur les cultures cellulaires en monocouche ont permis de confirmer la pénétration très rapide de l'Ola-FL dans les noyaux des trois lignées cellulaires TN MDA-MB-231, SUM1315 et HCC1937, ainsi que sa fixation sur sa cible cellulaire grâce aux tests de compétition. Par ailleurs, les tests réalisés en *end-point* à partir des sphéroïdes ont mis en évidence une rapide pénétration du composé dans toute la profondeur des sphéroïdes. De plus, sur les modèles de sphéroïdes présentant des gradients d'hétérogénéité cellulaire (sphéroïdes âgés de 14 jours), l'Ola-FL gardait une distribution homogène, même dans les zones hypoxiques.

Ces résultats permettent de valider l'utilisation de ce marqueur fluorescent dans les expériences précliniques utilisant les cultures cellulaires 3D. En effet, l'étude des anti-PARPs en développement par microscopie à fluorescence au sein des sphéroïdes, ouvre une perspective d'optimisation de leur efficacité, notamment en permettant de définir leur chimiorésistance associée.

E) Développement d'une méthode de quantification intracellulaire de l'anti-PARP Olaparib par Chromatographie Haute Performance en Phase Liquide (HPLC)

I) Introduction

A l'instar de la pénétration et de la distribution des anti-PARPs dans les tumeurs, leur concentration intracellulaire est un paramètre important permettant de définir son efficacité. Ainsi, les expériences réalisées avec les pharmaco-chimistes de l'équipe ont visé à développer une méthode simple, rapide et normalisée de quantification intracellulaire de l'Olaparib par Chromatographie Haute Performance en Phase Liquide (HPLC) couplée à un détecteur UV en barrettes diode (UV/DAD). Pour ce faire, un certain nombre de paramètres ont été optimisés en termes de préparation d'échantillon cellulaire et d'étapes de détection.

II) (*Article paru*) Development and validation of a high-performance liquid chromatography method for the quantitation of intracellular PARP inhibitor Olaparib in cancer cells.



Development and validation of a high-performance liquid chromatography method for the quantitation of intracellular PARP inhibitor Olaparib in cancer cells

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ABSTRACT

Olaparib is a potent PARP inhibitor in clinical use for cancer therapy. A bioanalytical assay was developed and validated for quantitation of intracellular level of olaparib in cells exposed to the drug. The assay involves an optimized and straightforward sample pretreatment with acetonitrile for olaparib solubilization, cell lysis and protein precipitation, and a high performance liquid chromatography (HPLC) method with ultraviolet detection. Several parameters in both the sample preparation and the detection steps were investigated. Optimal chromatographic conditions were achieved with a 5 μ L injection on a Nova-Pak[®] C₁₈ column (150 $\times$ 3.9 mm, 4 μ m) using a mobile phase consisting of acetonitrile and ultra-pure water in gradient mode, at a constant 1.2 mL/min flow rate, at 35 °C. Detection was carried out at 254 nm and a diode array detector was used to insure purity of the olaparib peak. The method was validated according to Food and Drug Administration guidelines. Linearity, accuracy and precisions were satisfactory over the concentration range of 200–2000 ng/mL. Limits of detection and quantification for olaparib were 50 ng/mL and 200 ng/mL, respectively. Good stability was showed in three relevant analytical conditions. Finally, the validated analytical method was successfully used to estimate the intracellular level of olaparib in SUM1315 breast cancer cells. A significant difference was observed in intracellular drug level after 1 and 3 h incubations. This method permitting measurement of drug level in tumor cells would allow dosage optimization and improvement of treatment response predictions.

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1. Introduction

Over the last decade, the inhibition of poly (ADP-ribose) polymerase (PARP), a nuclear enzyme involved in many processes including DNA repair and cell death, has become a promising personalized therapeutic strategy in cancer treatment [1–4]. PARP inhibitors, a new class of antineoplastic drugs, can induce tumor-specific synthetic lethality in cancer cells with defective DNA damage repair system, like those associated to mutations of the BRCA tumor suppressor genes [5–8]. Olaparib (AZD2281, Fig. 1),

veliparib (ABT-888), niraparib (MK-4827) or rucaparib (AG014699) are examples of potent PARP inhibitors having recently reached advanced clinical trials as combination and/or standalone targeted therapies, especially in breast and ovarian cancers. Olaparib (Lynparza) was the first to gain the European Commission (2014) and the United States Food and Drug Administration (2015) regulatory approvals for use in patients with BRCA-mutated ovarian cancer [5,9,10].

PARP inhibitors have great therapeutic potential and are anticipated to have broad clinical application in future cancer therapy [10]. However, both preclinical and clinical studies have revealed that tumor cells sensitivity towards PARP inhibitors can vary widely, and that treatments efficacy needs optimization. PARP being an intracellular target, the sensitivity of tumor cells and the effectiveness of a PARP targeted therapy are influenced by a key factor, namely the amount of PARP inhibitors reaching

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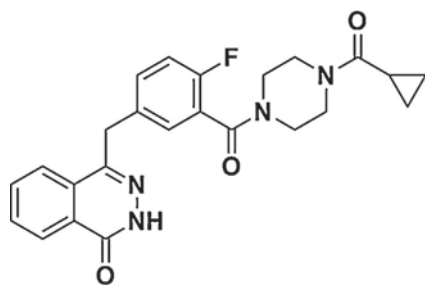


Fig. 1. Chemical structure of the PARP inhibitor olaparib.

the intracellular compartment [11]. As for any drug with an intracellular target, the disposition of PARP inhibitors is greatly affected by processes such as elimination, metabolism, drug uptake, and expression/upregulation of transmembrane drug efflux transporters. The latter, particularly relevant for PARP inhibitors, was identified during early preclinical studies as a major resistance mechanism [3,12–14].

In this context, methods that provide measurements of PARP inhibitors concentration, especially in tumor cells, have potential for drug dosage optimization and improvement of drug response predictions. Overall, such data would lead to the implemented therapeutic potential of PARP inhibitors. Therefore, a reliable methodology to assess intracellular level of PARP inhibitors in cancer cells is warranted, and could ultimately provide a mean to assess relationships between PARP inhibitors intracellular level and treatment efficacy.

Measuring intracellular concentration of a drug is challenging and has relied most of the time on the use of radiolabeled analogues. However, the main limitation of this methodology originates in the fact that such compounds are not readily available [15,16]. Mass spectrometry is a very sensitive and specific technique to determine drug levels in cells [16,17]. Accordingly, liquid chromatography-tandem mass spectrometry (LC–MS/MS) is the method of choice for low drug level detection but still requires high-cost instruments and time-consuming sample preparations. Regarding PARP inhibitors, LC–MS/MS methods were only reported for quantitative measurements in cell culture medium, human plasma, tumor and brain homogenates [19–23]. Oplustilova et al. recently mentioned the intracellular level determination of an olaparib analogue by LC–MS but no technical details were given on the analytical method that was employed [24]. Finally, flow cytometric analysis was used recently to perform single-cell analysis and assess intracellular concentration of fluorescent drugs [11,18,25,26]. Although promising, the development of this technique is still at an early stage and the low number of drugs with intrinsic fluorescence represents a major limitation. Reversed-phase high performance liquid chromatography (HPLC) with Ultraviolet Diode Array Detector (UV–DAD) analyses allow for sensitive quantitation of many compounds with accessible equipment. However, to the best of our knowledge, no validated method based on this technique for the determination of intracellular drug was ever reported in the literature.

The aim of the present study was to develop a simple, sensitive and reliable HPLC–UV–DAD procedure for quantitation of intracellular PARP inhibitor levels. The assay provides the advantage of working with un-labeled compounds and includes a straightforward and reliable cell sample pretreatment developed by varying the nature and the volume of the solvent used for solubilization of intracellular drug. The optimized analytical method was validated according to international guidelines for bioanalytical method validation and applied to the estimation of olaparib intracellular level in SUM1315 breast cancer cells.

2. Material and methods

2.1. Chemicals and reagents

Olaparib was purchased from Sequoia Research Products (Pangbourne, UK). All chemicals were acquired from Sigma-Aldrich (France). Dimethyl sulfoxide (DMSO) was of molecular biology grade. Water and acetonitrile were of HPLC gradient grade (VWR Chemicals, France).

2.2. SUM1315 cell culture

SUM1315 cells were obtained from Asterand (Royston, UK) and maintained by weekly serial passage in a 5% CO₂ atmosphere at 37 °C. Cells were cultured in Ham's F12 medium (GIBCO™, France) supplemented with 5% fetal bovine serum, 10 mM HEPES buffer, 20 µg/mL gentamicin, 10 ng/mL EGF and 4 µg/mL insulin [27].

2.3. Stock solutions, calibration standards and quality control samples

A 1 mg/mL stock solution of olaparib was prepared by dissolving an appropriate amount of olaparib in DMSO. By dilution of this solution with acetonitrile, separate 10000 ng/mL stock solutions for calibration standards and quality control samples (QC samples) were prepared and stored at –20 °C. Both stock solutions were further diluted to obtain working solutions. Calibration standards were prepared daily before each validation run. These were prepared by adding the appropriate amount of the 10000 ng/mL stock solution to acetonitrile to obtain concentrations of 200, 400, 600, 800, 1000 and 2000 ng/mL for olaparib. QC samples were prepared in a similar manner to obtain olaparib spiked solutions in acetonitrile used for sample preparation. Final olaparib concentrations at QC-low, QC-mid and QC-high were 300, 800 and 1800 ng/mL, respectively.

2.4. Chromatographic equipment and conditions

Analytical HPLC experiments were performed on a HP 1100 series LC system equipped with an online degasser, a quaternary pump, a column oven, a photodiode array detector (DAD) and an autosampler. Water and acetonitrile (ACN) used for the preparation of the mobile phase were filtered through a 0.45 µm Durapore Polyvinylidene difluoride (PVDF) membrane filter (Merck Millipore, UK) before being used in the chromatographic system. The temperature of the column oven was kept at 35 °C. A gradient elution was applied at a flow rate of 1.2 mL/min through a reversed-phase Nova-Pak® 4 µm C₁₈ column (150 × 3.9 mm; Waters Corporation, Milford, MA, USA) with an additional Nova-Pak® guard column. The following gradient was employed: 0–13 min, 5–35% ACN; 13–16 min, 35–95% ACN; 16–21 min, 95% ACN; 21–22 min, 95–5% ACN; 22–28 min, 5%. A sample volume of 5 µL was injected. UV spectra were recorded from 210 to 400 nm from 0 to 14 min and the monitoring wavelength for olaparib was set to 254 nm. Agilent ChemStation software (version B.04.03-SP1, Agilent Technologies, France) was used for acquisition of chromatograms, spectra and integration data.

2.5. Sample preparation

SUM1315 cells were seeded at a density of 250 000 cells/mL in 5 mL of culture medium and incubated at 37 °C during 24 h for cell adhesion to the flask. The culture medium was removed. Cells were subsequently washed with ice-cold PBS (2 × 2 mL) and then lysed by scraping in acetonitrile (2 mL) in conjunction with sonication (5 × 3 s). After centrifugation of the samples at 4 °C (350 g, 10 min),

the supernatant was filtered through a 0.45 μm PVDF membrane filter and pipetted into a 1 mL autosampler vial for injection.

2.6. Bioanalytical method validation

The described method was validated according to internationally accepted recommendations for bioanalytical method validation [28].

2.6.1. Selectivity

Six different cell samples processed without olaparib were analyzed for peaks of substances interfering with that of olaparib at 10.1 min. The chromatograms were compared to those obtained with the 200 ng/mL calibration standards and to blank samples (HPLC gradient grade acetonitrile).

2.6.2. Carry over effect

Carry over was investigated by injecting a blank sample (HPLC gradient grade acetonitrile) after each QC-high sample injection in each analytical run.

2.6.3. Recovery

The recovery of olaparib over the whole process from cell samples was determined using QC samples (QC-low, QC-mid, QC-high) and olaparib standard solutions in acetonitrile at the three validation levels (QC-low, QC-mid, QC-high). The recovery was calculated by comparing olaparib peak areas from QC samples ($n = 12$ for each concentration level) submitted to sample preparation (cell scraping and sonication, centrifugation, filtration) with the corresponding mean peak area of the unprocessed standard solutions ($n = 4$).

2.6.4. Calibration curves

The 200 and 2000 ng/mL calibration standards were processed in duplicate for each daily calibration, whereas the levels in between were processed only once. Linear regression analysis was employed to define the calibration curves using the olaparib peak area versus the nominal concentration of the calibration standard (ng/mL). For each analytical run, the calibration curve was accepted if all calibration standards had a deviation within $\pm 15\%$ of the nominal concentration. A deviation of $\pm 20\%$ for the 200 ng/mL calibration standards was accepted. Subsequently, the determined linear regression formula was used to measure concentrations of QC and unknown cell samples after integration of the olaparib peak area on the corresponding chromatogram.

2.6.5. Precision and accuracy

QC samples were used to assess the precision and the accuracy of the method. Each QC sample was processed in a quintuple analysis in four analytical runs on four separate days. The relative standard deviation (RSD) was calculated to determine the within day precision (repeatability) and the between day precision (reproducibility). The deviation of the mean measured concentration from the nominal value in percentage (bias%) was calculated to determine the within and between day accuracies.

2.6.6. Limit of quantification and limit of detection

The sensitivity was evaluated by determining the limit of quantification (LOQ), defined as the lowest concentration of the standard curve that can be measured with acceptable inter- and intra-day precisions and accuracies. RSD values lower than 20% and bias values within $\pm 20\%$ were considered acceptable for precision and accuracy, respectively.

The limit of detection (LOD) is defined as the lowest concentration of a compound that an analytical process can reliably differentiate from background level. LOD was determined by visual

evaluation of chromatograms of standards on which olaparib could be reliably detected.

2.6.7. Stability

The stability of olaparib in acetonitrile was investigated in QC-low and QC-high samples stored in HPLC glass vials. Quadruplicate analysis of these samples was performed after storage at room temperature (20 °C) for 24 h after thawing, three freeze-thaw cycles (thawing at room temperature during 12 h and freezing again at -20°C for at least one day), and storage at -20°C for one month.

2.7. Method applicability: determination of intracellular concentrations of olaparib in SUM1315 cells

SUM1315 cells were seeded at a density of 250 000 cells/mL in 5 mL of culture medium and incubated at 37 °C during 24 h for cell adhesion to the flask. The cell cultures were then exposed to 50 μM olaparib. For each experiment, the required volume of a 50 mM olaparib stock solution was added so that the final DMSO concentration remained always constant i.e. 0.1%. In parallel, untreated cells were cultured as control. The intracellular concentration of olaparib in SUM1315 cells was evaluated after 1 and 3 h of 50 μM olaparib exposures. After each incubation time, the culture medium was removed. Cells were subsequently washed with ice-cold PBS ($2 \times 2\text{ mL}$) and then lysed by scraping in acetonitrile (2 mL) in conjunction with sonication ($5 \times 3\text{ s}$). After centrifugation of the samples at 4 °C (350 g, 10 min), the supernatant was filtered through a 0.45 μm PVDF membrane filter and pipetted into a 1 mL autosampler vial for injection. The intracellular concentration of olaparib in cells was subsequently determined by HPLC-UV analysis. All experiments were performed in triplicate ($n = 3$) and each sample was analyzed three times. Statistical analyses were performed with Microsoft Excel using a two-sided Student's t -test with $p < 0.05$ considered significant.

3. Results and discussion

3.1. Method development

The aim of the present study was to develop and validate a simple and sensitive reversed-phase HPLC method with UV-DAD detection for quantitation of intracellular PARP inhibitor levels.

Starting from cells in culture flasks, the method reported herein relies on a straightforward sample pretreatment. For each cell sample, the culture medium was first removed and a wash step was performed in ice cold PBS in order to avoid disturbance in drug equilibrium. Indeed, the low temperature limits active transport of intracellular drug out of the cells during pretreatment. By analyzing the washing solution by HPLC, it was shown that a single wash was not sufficient to ensure the total elimination of the remaining extracellular drug in the culture medium and on the cell surface. For each sample, the wash was repeated twice with 2 mL of PBS, a volume which was kept minimal but was sufficient to cover the whole surface of the flask.

With the goal of developing an assay with limited manipulations, the next step implied to identify an organic solvent or a solvent mixture adapted to (i) lyse the cells when associated to sonication, (ii) solubilize intracellular olaparib, and (iii) allow precipitated proteins and cell debris to be easily eliminated by centrifugation. Acetonitrile and methanol were tested pure and as a 50/50 (v/v) mixture. A volume of 2 mL was chosen. Each trial gave a different result regarding cell structure. Indeed, after a very short time in contact with the cells, it was found by optical microscopy examination that pure methanol and the acetonitrile/methanol mixture led to fixation of the cells. Acetonitrile gave satisfactory results and was therefore chosen for this critical step. Subsequently,

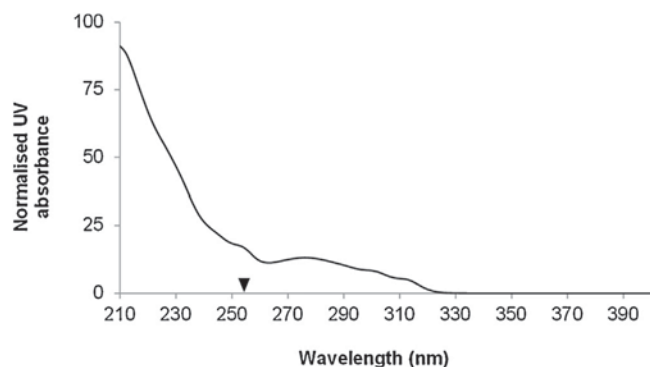


Fig. 2. UV absorption spectrum of olaparib (the black arrow indicates 254 nm, the wavelength used to monitor olaparib).

a simple filtration of the supernatant through PVDF membrane filters directly in HPLC vials provided the samples to be analyzed. A volume of 2 mL chosen after flask surface consideration allowed samples to be sufficiently concentrated for HPLC-UV detection, thus avoiding concentrating and reconstitution steps before analysis. HPLC injectable samples were obtained from cell cultures after a short time and a limited number of steps.

With regards to the chromatographic conditions, a usual UV-DAD detection system and standard chromatography elution conditions were employed with a general purpose Nova-Pak[®] C₁₈ column equipped with an additional Nova-Pak[®] guard column. A water-acetonitrile mobile phase with a gradient elution was tested. The gradient was optimized by varying the acetonitrile percentage in the initial mobile phase from 5 to 20% and the gradient slope. A low 5% acetonitrile starting mobile phase with a slow increase to 35% over 13 min was necessary for olaparib to be sufficiently retained on the column while keeping a reasonably short retention time. The choice of the detection wavelength was crucial for the sensitivity and selectivity of the method. A compromise was found and the detection wavelength was set to 254 nm, which corresponds to a maximum on the UV absorption spectrum of olaparib (Fig. 2). Although a better sensitivity could have been reached at a lower wavelength, too many peaks from co-eluting endoge-

nous substances were observed when recording chromatograms at 210 nm for selectivity assessment.

The use of acetonitrile during the sample pretreatment offers the opportunity of a small volume direct injection in the chromatographic system. Initially, broad peaks were observed even with injection volumes as low as 5 μ L. Therefore, the influence of two parameters was investigated to optimize peak shapes: the mobile phase flow rate was tested at 0.8 and 1.2 mL/min, and different temperatures of the column oven were applied, every 5 °C from 25 to 45 °C. Eventually, sharp and symmetric peaks were obtained with the following optimal conditions: 1.2 mL/min for the mobile phase flow rate and 35 °C for the oven temperature. In these optimized chromatographic conditions, olaparib was detected at a retention time of 10.1 min, for a 28 min total run time, including the column equilibration delay.

3.2. Method validation

3.2.1. Selectivity and carry over effect

Control SUM1315 cell samples processed without olaparib were analyzed under the chosen optimized chromatographic conditions to investigate the selectivity of the method. The chromatograms showed several peaks from endogenous substances but revealed no interference at the retention time of olaparib (10.1 min), indicating that the described method is selective. Typical HPLC chromatograms of a control cell sample processed without olaparib, a blank sample of gradient grade acetonitrile, a standard solution of olaparib in acetonitrile, and a QC-high sample are shown on Fig. 3a–d, respectively. The control cell sample chromatogram (Fig. 3a) showed peaks of substances eluting before or after olaparib but no peak was observed at 10.1 min. The blank sample chromatogram (Fig. 3b) showed no peak and a flat baseline, and finally a well resolved peak with symmetrical shape corresponding to olaparib appears at 10.1 min on both chromatograms 3c and 3d. For each sample in which olaparib was detected, the UV-DAD detector was used to assess the purity of the peak at 10.1 min by comparing the corresponding UV absorption spectrum (210–400 nm) with that obtained with a standard sample of olaparib. A perfect match was observed for each sample, which also indicates a good selectivity. A typical HPLC chromatogram of a cell sample treated with

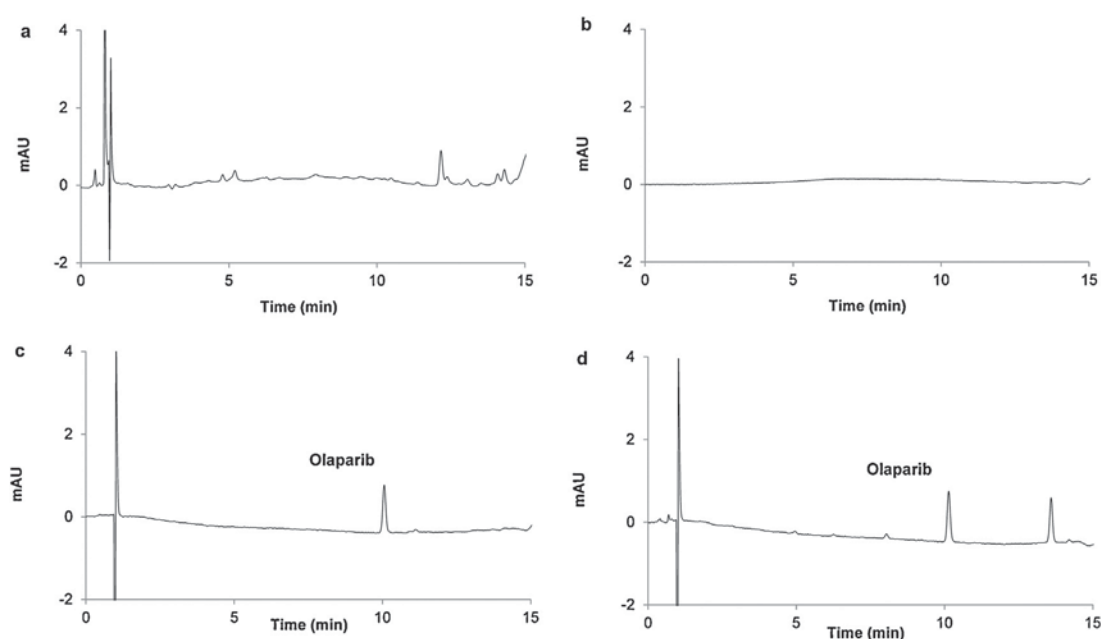


Fig. 3. Typical HPLC chromatograms of a control cell sample (a), a blank sample (b), a standard solution of olaparib in acetonitrile (1800 ng/mL) (c), and a QC-high sample (d).

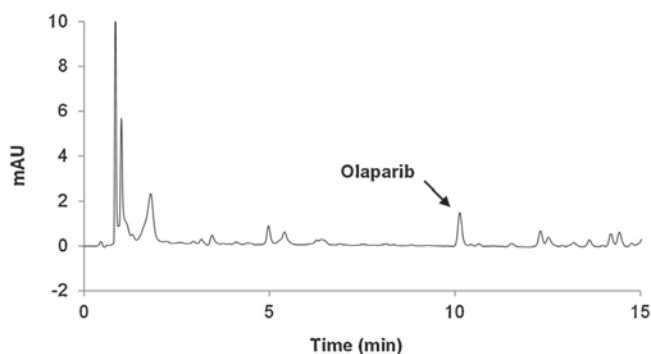


Fig. 4. Typical HPLC chromatogram of a cell sample treated with olaparib 50 μ M for 3 h.

Table 1
Recovery (%) of olaparib.

Concentration (ng/mL)	Recovery ^a (%)
QC-low (300 ng/mL)	99.0 $\pm$ 8.5
QC-mid (800 ng/mL)	99.7 $\pm$ 3.7
QC-high (1800 ng/mL)	101.2 $\pm$ 3.3

^a Mean $\pm$ standard deviation, $n = 12$.

olaparib 50 μ M for 3 h is shown on Fig. 4, with the corresponding well resolved peak appearing at 10.1 min. No carry-over effect was observed when blank samples were analyzed after QC-high samples injections.

3.2.2. Recovery

The recoveries of QC samples over the sample preparation process were calculated as the % ratio of olaparib peak area from processed QC samples and unprocessed standard solutions in acetonitrile. Percentages are shown in Table 1. Recoveries were quantitative at QC-low, QC-mid and QC-high levels. As no analyte losses were involved in our sample preparation, the use of internal standard was not necessary. Moreover, representative chromatograms of a standard solution of olaparib in acetonitrile (Fig. 3c) and a QC-high sample (Fig. 3d) presented the same profile. These results proved the relevance of using olaparib standard solutions in acetonitrile to construct calibration curves.

3.2.3. Calibration curves

The linearity of the method was then evaluated. Olaparib standard solutions in acetonitrile over the 200–2000 ng/mL concentration range were used to construct calibration curves, as quantitative recoveries were obtained with QC samples over the 300–1800 ng/mL concentration range. Linear relationships were established from olaparib standard solutions over the 200–2000 ng/mL concentration range, by plotting the olaparib peak area versus the corresponding concentration in ng/mL. Concentrations of all standards were back calculated from the olaparib peak area using the calibration curve in which they were included. In every case, deviations within $\pm 15\%$ of the nominal concentration

were found ($\pm 20\%$ at the LOQ, defined as the lowest concentration of the standard curve that can be measured with acceptable inter- and intra-day precisions and accuracies). The average regression parameters of the linear regression function ($n=4$) were $y=0.0050(\pm 0.0002)x+0.41(\pm 0.13)$. All calibration curves had correlation coefficient (r^2) of 0.997 or better. For the method described herein, the LOD was defined as 50 ng/mL. Visual evaluation of standards chromatograms on which olaparib could be reliably detected and distinguished from the background noise was chosen among other accepted approaches for the determination of the LOD. The sensitivity of the method was evaluated by LOQ determination. For the current assay, the LOQ for olaparib was set to 200 ng/mL, the lowest concentration that could be measured with acceptable precisions and accuracies following international recommendations. Lower values of LOQ were previously reported for the quantitation of olaparib but only LC-MS/MS methods were employed for such measurements, in cell culture medium, plasma, and tumor and brain homogenates [19,20,22,23]. With a LOQ of 200 ng/mL, the current HPLC-UV was sufficiently sensitive and was successfully applied to the intracellular olaparib level determination in SUM1315 cells after various treatment times. The 254 nm detection wavelength was indeed chosen during the method development as a compromise to obtain sufficient sensitivity while keeping an excellent selectivity.

3.2.4. Precision and accuracy

Precision and accuracy of the current method were evaluated using three QC samples at concentrations ranging from 300 to 1800 ng/mL. Samples were processed in a quintuple analysis in four analytical runs on four separate days. Precision and accuracy values are summarized in Table 2. The within- and between-day precisions, expressed as RSD (%), were below 11% at all QC levels. Similarly, the within- and between-day accuracies, expressed as bias (%), were below 13% at all QC levels. Precision and accuracy therefore met the $\pm 15\%$ required for method validation by the U.S. Food and Drug Administration [28].

3.2.5. Stability

Stability studies were also performed to ensure good reproducibility of the method. The stability of olaparib in acetonitrile was evaluated in different conditions on high and low QC samples. Tested conditions included short-term room temperature and long-term low temperature storage. For short-term stability, QC samples were thawed and kept for 24 h at room temperature before analysis. Long-term stability was evaluated after a 30-day storage period at -20°C . Finally, data obtained after three freeze-thaw cycles was also collected. Results are shown in Table 3. No variation higher than 8.4% or 3.2% of the initial measured concentration was found for QC-low and QC-high samples, respectively. Hence, these results indicated that no stability issue would be expected after short or long term storage of the samples for quantification studies.

Table 2
Within and between day precision and accuracy of the HPLC analytical method, expressed as RSD and bias percentages, respectively.

Nominal concentration (ng/mL)	Within day ^a			Between day ^b		
	Experimental concentration (ng/mL)	RSD (%)	Bias (%)	Experimental concentration (ng/mL)	RSD (%)	Bias (%)
300	315 $\pm$ 18	5.71	4.84	288 $\pm$ 21	7.18	−4.06
800	789 $\pm$ 19	2.40	−1.38	765 $\pm$ 45	5.92	−4.35
1800	1737 $\pm$ 34	1.97	−3.53	1762 $\pm$ 101	5.74	−2.09

^a Mean $\pm$ standard deviation, $n = 5$.

^b Mean $\pm$ standard deviation, $n = 20$.

Table 3

Stability of olaparib in acetonitrile (percentages of the initial measured concentration).

Condition	QC-low ^a (%)	QC-high ^a (%)
24 h at ambient	108.4 ± 7.5	96.8 ± 3.6
3 freeze/thaw cycles	95.0 ± 3.0	101.3 ± 1.7
1 month at −20 °C	93.3 ± 5.3	97.0 ± 1.8

^a Mean ± standard deviation, *n* = 4.

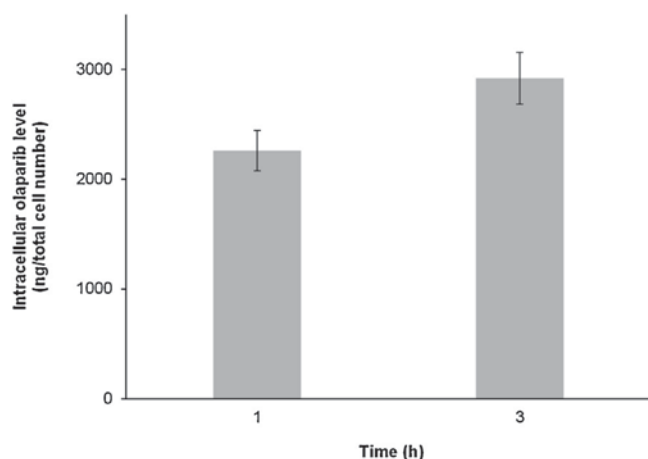


Fig. 5. Intracellular olaparib concentrations in SUM1315 cells treated with 50 μ M olaparib for 1 and 3 h (*n* = 3).

4. Method applicability

The described method was developed to assess the intracellular level of a PARP inhibitor in cultured cells. A suitable sensitivity for standard cell culture conditions was achieved while limiting the number of steps during the sample pretreatment, namely culture medium removal, repeated wash step in ice-cold PBS, solubilization and sonication in acetonitrile, centrifugation for precipitated proteins elimination, and PVDF membrane filtration. Notably, no time-consuming sample concentration by solvent evaporation and subsequent reconstitution step are required. The described method was then used to determine the intracellular concentration of olaparib, a clinically relevant PARP inhibitor, in SUM1315 breast cancer cells after a 50 μ M treatment. Olaparib was detected in cell samples and the corresponding peak was integrated for quantitative analysis (Fig. 4). Results, expressed in ng/total cell number, are shown in Fig. 5. Olaparib level in cells increased significantly ($p < 0.05$) from 1 to 3 h, which is in accordance with previous results [14]. Therefore, the described method allowed the monitoring of intracellular level of olaparib over time. Such data will be of great interest when considering the optimization of PARP inhibitor-based treatment efficacy.

5. Conclusion

In the present report, a simple, selective and sensitive reversed-phase HPLC method with UV detection was developed for determining PARP inhibitors intracellular concentration, and validated according to internationally accepted recommendations for bioassay validation. Importantly, the equipment required is readily available and much less expensive than other quantitative techniques such as tandem mass spectrometry. The proposed method has also the advantages of being straightforward and rapid, so that it was successfully applied to the quantitation of intracellular PARP inhibitor olaparib in SUM1315 breast cancer cells at various times of treatment. This analytical method could be extended to other

cell lines and other PARP inhibitors to obtain valuable data for the successful prediction of drug efficiency in cancer treatment.

Conflict of interest

The authors declare that they have no conflict of interest.

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III) Conclusion sur les travaux

Ces travaux ont permis la détermination des conditions (i) de préparation des échantillons cellulaires et (ii) des paramètres de détection optimaux pour le dosage intracellulaire de l'Olaparib. En effet, la sensibilité, la précision et la reproductibilité de la méthode ont obtenu des résultats très satisfaisants. Ainsi, cette méthode peut être utilisée dans l'arsenal des outils permettant la caractérisation de la pénétration / distribution des anti-PARPs dans les cellules tumorales, en corrélation avec leur efficacité.

F) Évaluation de l'expression des protéines de MultiDrug Resistance MRP7 et P-gp suite au traitement par Olaparib sur des modèles de culture 2D et 3D.

I) Introduction

Malgré des résultats prometteurs dans les essais précliniques et cliniques, certaines résistances aux traitements anti-PARP ont été mises en évidence au niveau moléculaire et / ou cellulaire. Parmi ces systèmes de résistance, la surexpression des pompes d'efflux Multi Drug Resistance (MDR) au sein des tumeurs a été identifiée comme étant l'un des obstacles majeurs à l'efficacité des traitements. Au cours de nos travaux antérieurs, une co-expression de type relais de deux protéines MDR majeures, la P-gp et la BCRP, a été identifiée chez la lignée SUM1315 du cancer du sein triple négatif, après un traitement avec 50 μ M d'Olaparib durant 24h. Dans ce contexte, nous avons orienté ces travaux sur la détermination de la co-expression de la P-gp et de MRP7, une autre protéine MDR d'intérêt, suite au traitement par Olaparib à court et long-terme, dans différents modèles de culture cellulaire TNBC, en conditions 2D et 3D.

II) (Article rédigé) MRP7 and P-gp chemoresistance proteins expression in MDA-MB-231 and SUM1315 spheroids following Olaparib treatment.

MRP7 and P-glycoprotein chemoresistance proteins expression in MDA-MB-231 and SUM1315 spheroids following Olaparib treatment.

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ABSTRACT

Among the multiple breast cancer subtypes, the Triple-Negative tumours (TNBC) are particularly aggressive and difficult to treat, leading to very poor prognosis. In order to optimize patient outcome, Poly-ADP-ribose Polymerase inhibitors (anti-PARP) targeted therapies were developed, such as the anti-PARP1 Olaparib. Although promising results in preclinical and clinical studies, some resistances to Olaparib were highlighted at the molecular or cellular level. Among them, the overexpression of Multi Drug Resistance (MDR) efflux pumps targeting anti-PARP and notably Olaparib, is a major obstacle for the treatments efficacy. Thereby, these works aimed to analyze the co-expression of MRP7 and P-gp, in two TNBC cell lines, MDA-MB-231 and SUM1315, cultured in 2D and 3D with Olaparib treatment. In MDA-MB-231 2D cell culture experiments, 5 and 50 μ M Olaparib treatment induced a relay-expression of MRP7 and P-gp proteins, at short-term (4h). In both spheroid models, after 5 μ M Olaparib treatment at long-term (8 to 10 days), P-gp expression increased whereas no MRP7 increase was detected. These results showed clearly the implication of these two major MDR proteins in TNBC cell lines resistance against Olaparib, suggesting the persistence of selected-resistant cells in spheroids after treatment.

KEYWORDS

Triple-Negative Breast Cancer, TNBC, Olaparib, Anti-PARP, Multi Drug Resistance protein, MRP7, P-gp, 3D cell culture

INTRODUCTION

INTRODUCTION

Breast cancer is a very complex and heterogeneous disease. Among the several molecular subtypes, the Triple-Negative phenotype (TNBC), representing 15-20% of breast cancers, is particularly aggressive (**Foulkes et al., 2010; de Ruijter et al., 2011**). These tumours are also frequently mutated in BRCA1 / BRCA2 genes or present a “BRCAness” phenotype (**Elstrodt et al., 2006; Tassone et al., 2003**). In order to improve the patient’s outcome, several specific targeted therapies such as Poly-ADP-ribose Polymerase inhibitors (PARPi), exploiting the “BRCAness status” of tumours by the synthetic lethality concept, were developed in preclinical and clinical studies (**Wang et al., 2018**). Nevertheless, some resistances to anti-PARP treatments were highlighted at the molecular and/or the cellular level (**Bugde et al., 2017**). Among these resistance systems, the overexpression of Multi Drug Resistance proteins (MDR) is a major obstacle for the treatment of cancer. Indeed, the MDR proteins belong to a family of transmembrane ATP-Binding Cassette (ABC) transporters, that are specifically responsible for the rapid efflux of a large panel of xenobiotics, principally hydrophobic and ionic amphipathic molecules such as chemotherapeutics (**Chen et al., 2003; Gottesman et al., 2002**). Thus, their overexpression in tumour cells leads to decreased intracellular drug concentrations and an eventually decreased toxicity. Thereby, the characterization of MDR proteins at the tumoral level is of critical importance to improve the cancer treatment effectiveness (**Munoz et al., 2007**). The first MDR protein to be identified was the P-glycoprotein (P-gp, coded by ABCB1/MDR1 gene)(**Bugde et al., 2017**). Alongside, other MDR proteins of clinical interest were discovered, such as (i) the Breast Cancer Resistance Protein (BCRP / ABCG2 gene), initially found on models of mammary tumors resistant to various chemotherapeutic molecules such as cisplatin or paclitaxel (**Hira and Terada, 2018**), (ii) the Multi-Drug Resistance protein 1 (MRP1/ABCC1 gene) and (iii) the MRP7 protein (ABCC10 gene)(**Munoz et al., 2007; Sun et al., 2013**).

In this context, these works aimed to determine the co-expression of MRP7 and P-gp after exposition to Olaparib in two TNBC cell lines *ie.* MDA-MB-231 and SUM1315 cultured in 2D and 3D cell cultures.

MATERIAL AND METHODS

1 Cell culture

Two Triple-Negative breast cancer cell lines, SUM1315 (Asterand®, MO2) and MDA-MB-231 (ATCC®, HTB-26), were used in this study. SUM1315 cell line was cultured in Ham's F12 medium (Gibco®) supplemented with 5% decompartmented fetal calf serum, 10 mM HEPES buffer (Sigma®), 20 µg/mL gentamycin (Panpharma®), 10 ng/mL EGF (Sigma®) and 4 µg/mL insulin (NovoRapid®). MDA-MB-231 cell line was cultured in RPMI medium 1640 (GIBCO®) supplemented with 10% decompartmented fetal calf serum and 20 µg/mL of gentamycin. Both cell lines were maintained at 37°C under 5% CO₂ in a humidified incubator (Sanyo®). For both lines, cells were seeded in 15 ml of culture medium at a concentration of 5000 cells / cm² in 75 cm² Falcon® culture flasks, following supplier's instructions. Before confluence, cells were trypsinized, counted with the blue trypan exclusion test and re-seeded into new culture flasks at the same cell concentration, or processed in our experiments. The cell lines were cultured until passage number 45. The absence of mycoplasma contamination was systematically checked before running the cell experiments with Mycoplasma Check kit (GATC®).

1.1 Cell culture in monolayer

Cells were seeded into Ibidi® cell-culture-treated microscopy chambers (Ibidi®) at a concentration of 5000 cells/cm² in 200 µL of medium. Cells were let in the incubator for 24 hours before the treatments in order to allow their attachment to the support.

1.2 Cell culture in three dimensions: spheroid formation with liquid overlay technique (LOT)

For SUM1315 and MDA-MB-231 cell lines spheroids formation, the liquid overlay technique was performed according to previous works of the team (**Dubois et al., 2017**). Cells were seeded into 96-well "Ultra-Low-Attachment" microplates (Corning®, 4520) at a concentration of 1000 cells/well in 100 µL of culture medium. 24h after cell seeding, a solution of cold Geltrex® was added to the culture medium for a final concentration of 2%. Plates were then shaken at 200 rpm for 20 min on a microplate agitator and then incubated for 24h until spheroid formation. Spheroid size follow-up was monitored with Cytation3®MV plate reader (Biotek®).

2 Cell culture treatment with Olaparib

Olaparib (Carbosynth®) was solubilized in 100% Dimethylsulfoxide (DMSO) at a concentration of 100 mM, constituting the stock solution. Olaparib was then diluted in the culture medium at final concentrations of 5 µM and 50 µM. For all tests, the final concentration of DMSO in the culture medium was of 0.1%. Cells cultured in monolayer were treated with 5 and 50 µM Olaparib for 4h. Spheroids were treated with 5 µM Olaparib for 8 or 10 days.

3 MRP7 and P-gp expression by immunofluorescence imaging

3.1 Monolayer cell culture

After Olaparib treatment, cells were fixed in cold Paraformaldehyde (PAF) 4% for 20 min, washed three times in PBS and incubated with a saturation solution for 1h (PBS/BSA 1%). Then, incubation with the primary antibody MRP7 (rabbit, polyclonal, Sigma, 1/250 in PBS/BSA 1%) or P-gp (mouse, clone F4, Invitrogen, 1/75 in PBS/BSA 1%) was performed for 30 min, followed by three washes of 5 min. Similarly, the isotype control was performed (XXX). Then, cells were incubated with the secondary antibody (anti-rabbit 568 Alexa Fluor, Molecular Probes, 1/800 in PBS/BSA 0.1%) for 30 min and with Hoechst 10 µg/ml for 5 min. Controls with no secondary antibody were also performed. Samples were imaged with Cytation3MV multimode fluorescence imager and the image analysis was performed with Icy® spot detector module. For the quantification of MRP7 and P-gp expression per cell, the following formula was processed:

$\text{Normalized fluorescence intensity per cell} = \frac{(\text{Number of spots per image} * \text{mean spot intensity per image})}{\text{cell number per image}}$
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3.2 3D cell culture

After Olaparib treatment for 8 or 10 days, spheroids were washed in PBS, fixed with PAF 4%, dehydrated with successive sucrose incubations (15 and 30%), quickly frozen in liquid nitrogen and cut with a cryostat (CICS, Clermont-Ferrand). Then, the similar protocol as for 2D cell cultures was performed for the MRP7 and P-gp markings. The slides were finally mounted with Prolong Diamond (Molecular Probes) and imaged with Cytation3MV plate reader.

The intensity of MRP7 or P-gp marking per spheroid and the number of cells per spheroid were determined with the *cellular analysis* algorithm of the Gen 5.03 software (Biotek®).

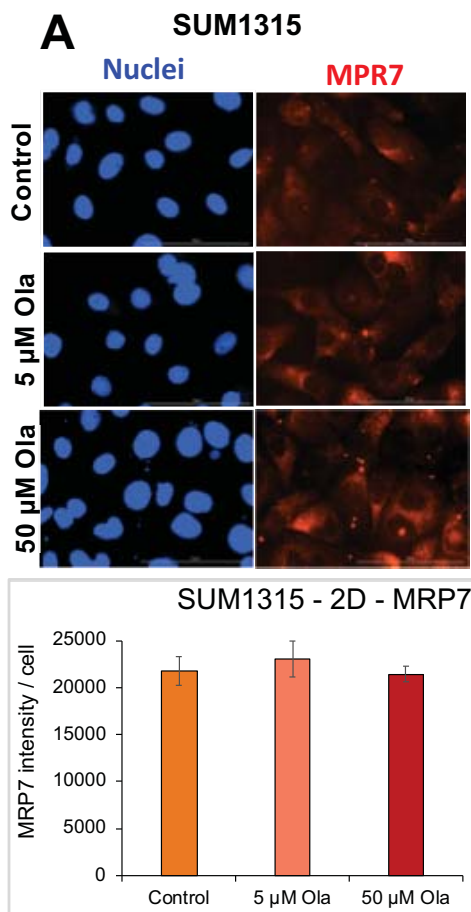
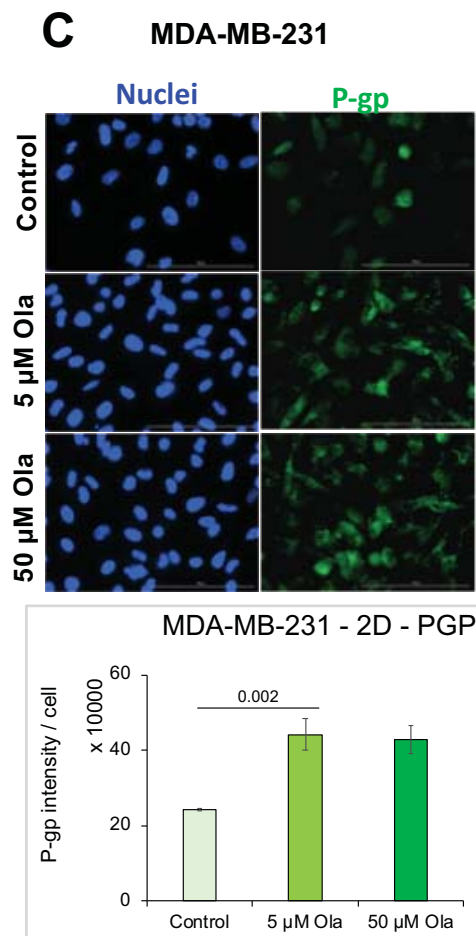
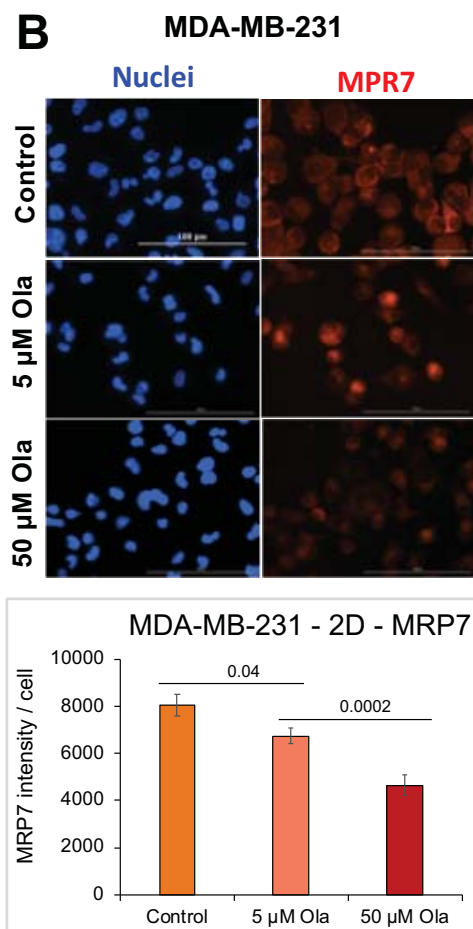


Figure 1: MRP7 and P-gp expression in SUM1315 (A) and MDA-MB-231 (B,C) TNBC cell lines following 4h Olaparib treatment. Cells were treated with 5 or 50 μ M Olaparib for 4h. Then, the cellular carpet was fixed with PAF 4%, saturated with 1% BSA and incubated with MRP7 or P-gp primary antibodies. After three washes in PBS, cells were then incubated with the appropriate fluorescent secondary antibodies. Images (> 5 for each condition) were acquired with Cytation3MV multimode plate reader. Results are expressed as mean MRP7 or P-gp fluorescence intensity normalized to the cell number with their standard error. Scale bar = 100 μ m.



Then, the normalized fluorescence intensity of MRP7 and P-gp per cell was calculated as follows:

$\text{Normalized fluorescence intensity per cell} = \frac{\text{Intensity of spheroid marking per image}}{\text{cell number per spheroid}}$
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4 Statistical analysis

Results were expressed as means $\pm$ standard deviation of *n* independent experiments. All experiments were statistically compared using a Student's *t*-test. Tests were two-sided, and the nominal level of significance was $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****), $p < 0.00001$ (*****) or *ns* non-significant ($p > 0.05$).

RESULTS

1 MRP7 and P-gp co-expression in MDA-MB-231 and SUM1315 cell lines cultured in monolayer after Olaparib treatment

The expression of MRP7 and P-gp proteins was firstly determined after 4h of treatment with 5 and 50 μM of Olaparib, on MDA-MB-231 and SUM1315 monolayer cell lines by immunofluorescence. The normalized fluorescence intensity of MRP7 and P-gp marking per cell (Fluorescence Unit: FU/cell) was measured with Cytation3MV imaging device (**Fig. 1**). For P-gp expression in SUM1315 cell line, our research team already highlighted the induction of expression in presence of 50 μM Olaparib (**Dufour et al., 2015**).

For SUM1315 cell line, the basal expression of MRP7 (control condition) was of 21732 ± 1548 FU/cell (**Fig. 1A**). Then, after a treatment with 5 and 50 μM Olaparib, this expression remained relatively stable with 23087 ± 1913 and 21433 ± 825 FU/cell, respectively ($p = 0.64$ and $p = 0.86$ compared to controls).

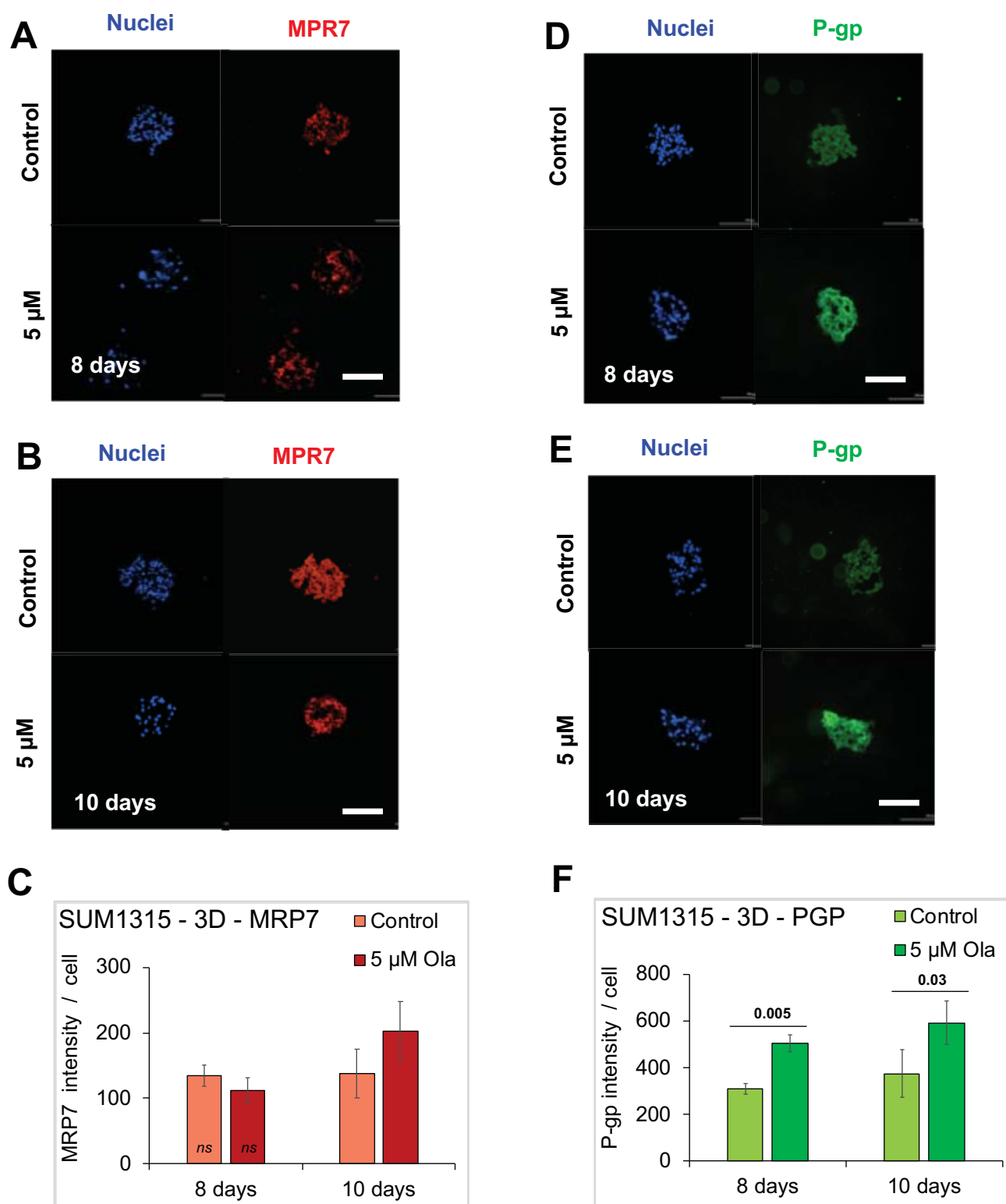


Figure 2: MRP7 (A, B, C) and P-gp (D, E, F) expression in SUM1315 spheroids after 5 and 50 μ M Olaparib treatment for 8 (A, D) and 10 days (B, E). Spheroids were treated with 5 μ M Olaparib for 8 and 10 days. Then, spheroids were fixed with PAF 4%, cut in ultrafin slides of 10 μ m with a cryostat. The slides were then saturated with 1% BSA and incubated with anti-MRP7 or anti-P-gp primary antibodies. After three washes in PBS, cells were then incubated with the appropriate fluorescent secondary antibodies. Images (> 10 spheroids cuts for each condition) were acquired with Cytation3MV multimode plate reader. Results are expressed as mean MRP7 or P-gp fluorescence intensity normalized to the cell number, with their standard error (C, F). Scale bar = 200 μ m

For MDA-MB-231 cell line, the basal expression of MRP7 was of 8045 ± 443 FU/cell. After 5 and 50 μ M Olaparib treatment, it decreased significantly to 6729 ± 331 FU/cell and 4650 ± 439 FU/cell, respectively ($p=0.04$ and $p=0.0002$ compared to control) (**Fig. 1B**). For P-gp expression, the, marking intensity was of 240683 ± 41800 FU/cell for the controls and increased significantly after 5 and 50 μ M Olaparib treatment to 440534 ± 36464 FU/cell and 428733 ± 29144 FU/cell, respectively ($p=0.002$ and $p=0.002$ compared to controls) (**Fig. 1C**).

All these results highlighted a different MRP7 and P-gp co-expression after a short-term (4h) Olaparib treatment, in SUM1315 and MDA-MB-231 cell lines cultured in 2D. Indeed, for whatever tested Olaparib concentration, MRP7 did not increase. In contrast, for MDA-MB-231, P-gp expression increased significantly in presence of 5 or 50 μ M Olaparib.

2 MRP7 and P-gp co-expression in TNBC spheroids following long-term treatment with low Olaparib concentration

For 3D cell cultures experiments, the low dose of 5 μ M Olaparib was chosen for the analysis of MRP7 and P-gp co-expression at long-term (8 and 10 days). The normalized fluorescence intensity of MRP7 and P-gp marking per cell (Fluorescence Unit: FU/cell) was analysed on MDA-MB-231 and SUM1315 spheroid cuts (**Fig. 2 and 3**).

For SUM1315 spheroids, the basal expression of MRP7 in spheroid controls remained constant after 8 and 10 days of culture with 135 ± 16 and 138 ± 38 FU/cell, respectively ($p=0.88$) (**Fig. 2A, B, C**). Then, in presence of 5 μ M Olaparib, MRP7 expression remained similar to controls after 8 days (112 ± 20 FU/cell, $p=0.16$) and 10 days of treatment (203 ± 45 FU/cell, $p=0.11$). Similarly, the basal expression of P-gp in SUM1315 spheroid controls was comparable after 8 or 10 days of culture with 309 ± 22 and 374 ± 102 FU/cell, respectively ($p=0.38$) (**Fig. 2D, E, F**). In contrast, after 5 μ M Olaparib treatment, P-gp expression increased significantly at day 8 with 502 ± 37 FU/cell ($p=0.005$) and remained similarly high compared to controls at day 10 with 593 ± 93 FU/cell ($p=0.03$).

For MDA-MB-231 spheroids, the MRP7 basal expression of spheroid controls was of 42 ± 9 FU/cell after 8 days of culture and remained stable after 10 days with 49 ± 19 FU/cell ($p=0.52$) (**Fig. 3A, B, C**). After treatment with 5 μ M Olaparib, this expression remained similar for the two treatment durations, with 67 ± 25 FU/cell after 8 days ($p=0.14$) and 70 ± 21 FU/cell after 10 days ($p=0.09$). Then, the P-gp expression in MDA-MB-231 control spheroids remained

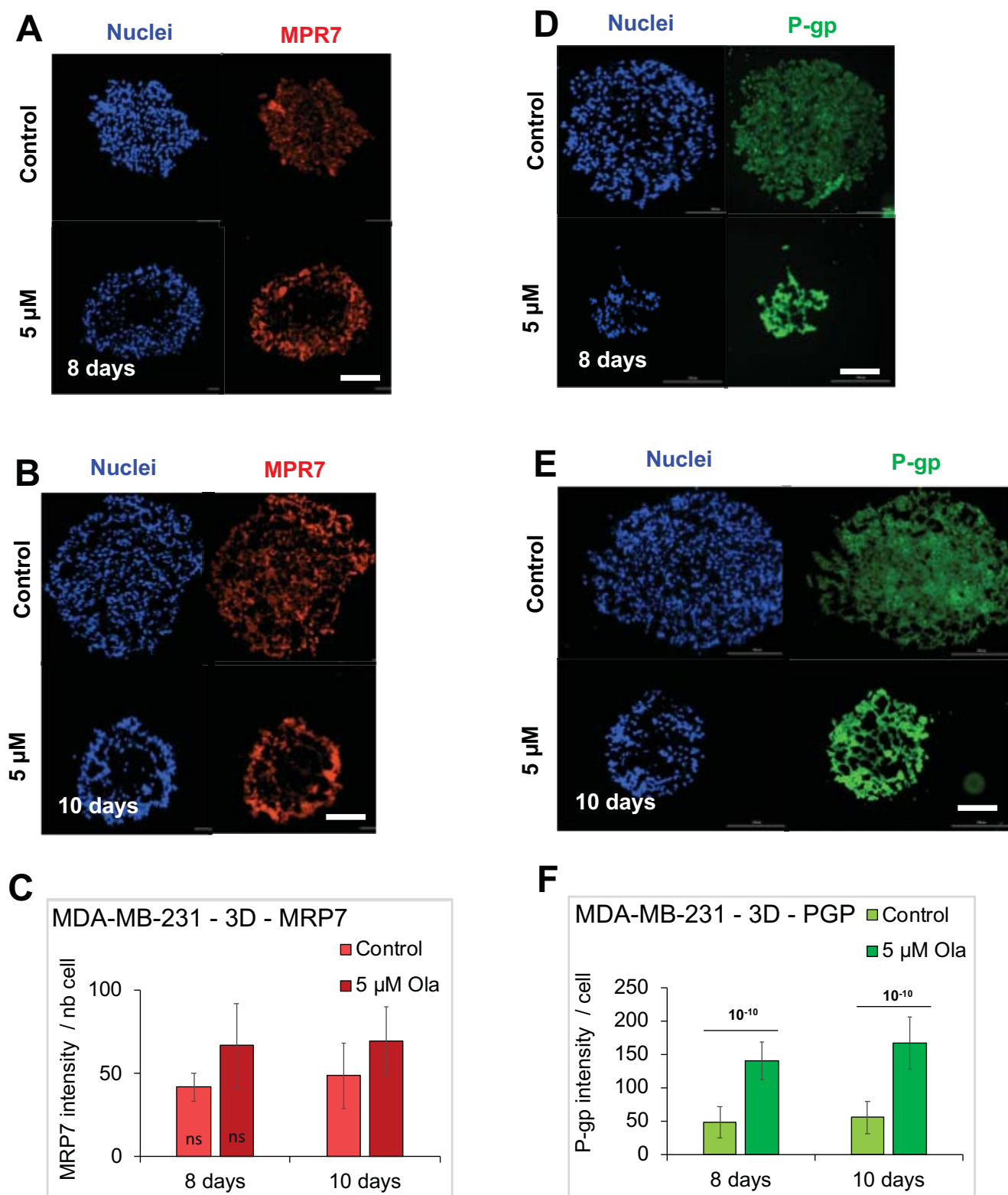


Figure 3: MRP7 (A, B, C) and P-gp (D, E, F) expression in MDA-MB-231 spheroids after 5 and 50 μ M Olaparib treatment for 8 (A, D) and 10 days (B, E). Spheroids were treated with 5 μ M Olaparib for 8 and 10 days. Then, spheroids were fixed with PAF 4%, cut in ultrafin slides of 10 μ m with a cryostat. The slides were then saturated with 1% BSA and incubated with anti-MRP7 or anti-P-gP primary antibodies. After three washes in PBS, cells were then incubated with the appropriate fluorescent secondary antibodies. Images (>10 spheroids cuts for each condition) were acquired with Cytation3MV multimode plate reader. Results are expressed as mean MRP7 or P-gp fluorescence intensity normalized to the cell number, with their standard error (C, F). Scale bar = 200 μ m

comparable after 8 days or 10 days of culture with 49 ± 23 FU/cell and 56 ± 24 FU/cell, respectively ($p=0.44$) (**Fig. 3D, E, F**). In contrast, after 5 μ M Olaparib treatment, the P-gp expression increased significantly with 141 ± 28 FU/cell ($p=10^{-10}$) and 167 ± 38 FU/cell ($p=10^{-10}$) after 8 and 10 days of culture, respectively.

All these results showed that the low dose at long-term Olaparib treatment induced a systematic overexpression of P-gp protein in both cell lines cultured in 3D. Conversely, no significant increase in MRP7 expression was detected after treatment.

DISCUSSION

Breast cancer is a very complex and heterogeneous disease. Among the several molecular subtypes, the Triple-Negative phenotype (TNBC), representing 15-20% of breast cancers, is characterized by the absence of progesterone receptor and estrogen receptor expression and Human Epidermal Growth Factor Receptor-2 (HER2) receptor overexpression (**Foulkes et al., 2010; de Ruijter et al., 2011**). These tumours are also frequently mutated in BRCA1 / BRCA2 genes or present a “BRCAness” phenotype (**Elstrodt et al., 2006; Tassone et al., 2003**). Because of their particularity, these tumours are not sensitive to hormone therapies or targeted therapies such as anti-HER2 and the only therapeutic strategy is conventional chemotherapy such as **FEC** (5-Fluorouracil, epirubicin, cyclophosphamide) or **FAC** (5-Fluorouracil, adriamycin, cyclophosphamide) protocols associated with taxanes regimen (**PDQ Adult Treatment Editorial Board, 2002**). Following treatment, unfortunately, a rapid and high number of relapses is encountered in 50% of patients leading to poor prognosis. Therefore, this subtype is particularly aggressive.

In order to improve the patient's outcome, several specific targeted therapies such as Poly-ADP-ribose Polymerase inhibitors (PARPi), exploiting the “BRCAness status” of tumours by the synthetic lethality concept, were developed in preclinical and clinical studies (**Wang et al., 2018**). Nevertheless, some resistances to anti-PARP treatments were highlighted at the molecular and/or the cellular level. Indeed, the generation of secondary mutations on BRCA1 or BRCA2 target genes, or the loss of the protein **53BP1** were identified as potential anti-PARP resistance biomarkers (**Dhillon et al., 2016; Rottenberg et al., 2008**). Moreover, the resistance mediated by the overexpression of Multi Drug Resistance proteins (MDR) is another major obstacle for the treatment of cancer.

Our former works with fluorescent derivative Olaparib molecule (Ola-FL) highlighted the rapid and homogeneous penetration / distribution of this PARPi in 2D and 3D MDA-MB-231 and SUM1315 cell cultures. The efficacy/toxicity of this treatment depends on its long-term maintenance in cells. Thus, Olaparib is a target molecule for MDR pumps efflux (**Dewanjee et al., 2017; Gameiro et al., 2017**). In this context, our works aimed to study the co-expression of two clinically relevant MDR proteins that are MRP7 and P-gp, in 2D and 3D culture conditions.

In the experiments performed in 2D cell culture, the short-term induction of both pumps was studied in order to determine MDR pumps induction following two doses of Olaparib treatment (5 and 50 μ M). On MDA-MB-231 cell line, the MRP7 expression decreased in a dose-dependent manner. Indeed, a 1.2 and 1.7-fold drop in MRP7 fluorescence intensity per cell was detected after 5 and 50 μ M, respectively. In contrast, a 1.8-fold increase in P-gp expression was detected on this cell line after both Olaparib treatment conditions. This might suggest a MRP7 / P-gp relay expression on MDA-MB-231 cells further to short-term Olaparib treatment. Indeed, MDR pumps can be sequentially or simultaneously overexpressed in tumors, in order to potentiate / optimize their efflux capacities. This was already demonstrated in our research team, where the relay-overexpression of P-gp and BCRP was detected further Olaparib treatment (50 μ M) on the TNBC SUM1315 cell line culture in monolayer (**Dufour et al., 2015**). In these latter works, BCRP was firstly overexpressed after 1h30 to 3h Olaparib treatment and then P-gp overexpression was detected from 3h30 to 24h. In these works, no induction of MRP7 overexpression was detected in SUM1315 cell line, suggesting an already saturated level of P-gp or BCRP proteins at the surface of the cancer cells.

Then, in the 3D cell cultures experiments, MRP7 and P-gp expression was studied after a long-term and low dose Olaparib treatment on MDA-MB-231 and SUM1315 spheroids. Indeed, our previous works demonstrated the efficacy of a long-term Olaparib administration (up to 10 days) in presence of relatively low doses (5 μ M compared to 50 μ M), on the same spheroid models. Thus, this treatment induced spheroid growth inhibition and cytotoxicity of about 30% to 60% depending on the cell line (***refers to the unpublished works described in section C of this manuscript***). In this study, for both cell lines, the MRP7 expression level remained comparable in the 5 μ M Olaparib treated spheroids compared to controls, and this after 8 or 10 days of treatment. Conversely, the P-gp expression was systematically increased in presence of Olaparib 5 μ M and this for the two spheroids models. Indeed, a 1.6-fold increase in P-gp expression was detected in SUM1315 spheroids and a 3.0-fold increase in MDA-MB-231 spheroids.

All these results obtained from 2D and 3D TNBC cell cultures suggest a relay-implication of the two clinically relevant MDR pumps such as P-gp or MRP7, in response to low doses of Olaparib. Nevertheless, MDR expression in 3D cell culture after the long-term Olaparib treatment (8 to 10 days) may be related to the remaining selected-resistant cells in spheroids. These results suggest the implication of these two major MDR proteins in TNBC cell lines resistance against Olaparib.

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AUTHOR CONTRIBUTIONS

C.D., P.D., C.A., E.M., F.P.L., M.B. conceived and designed the study;

C.D., P.D., M.D. performed the experimental work;

C.D., M.B., E.M. analyzed the data;

C.D., P.D., M.B. wrote the manuscript. All authors reviewed the manuscript.

ADDITIONAL INFORMATION

Competing interests: The authors declare no competing financial interests.

III) Conclusion sur les travaux

L'analyse de la co-expression sur les cultures 2D de la lignée MDA-MB-231, des deux pompes MDR d'intérêt clinique, la MRP7 et la P-gp, a permis de démontrer une expression de type relai après un traitement avec 5 et 50 μ M l'Olaparib pendant 4h. Pour la lignée SUM1315, aucune modification de l'expression de MRP7 n'a été mise en évidence suite au traitement. Ceci peut être expliqué par une réponse de type relai de la BCRP et de la P-gp déjà engagée dans les cellules de cette même lignée dans les mêmes conditions expérimentales, déjà démontrée au cours des travaux antérieurs de l'équipe (**Dufour, 2015**). Parallèlement, une analyse de la co-expression de la MRP7 et de la P-gp ont été effectuées à partir des cultures cellulaires 3D traitées avec 5 μ M d'Olaparib, après 8 à 10 jours de traitement. Après un traitement à long-terme avec une faible dose d'Olaparib, l'expression basale de la MRP7 reste inchangée alors qu'une nette induction de l'expression de la P-gp est détectée dans les cellules résiduelles persistantes dans les sphéroïdes, suite au traitement avec cet anti-PARP. Ces résultats suggèrent une implication de la protéine P-gp dans la résistance au traitement par Olaparib à long-terme avec l'Olaparib administré seul et à faible dose.

L'ensemble de ces résultats montrent d'une part l'expression de type relai de différentes pompes MDR majeures dans les deux lignées SUM1315 et MDAMB-231 après traitement avec l'Olaparib à court et à long-terme. Des études complémentaires de l'activité de ces pompes MDR en présence d'inhibiteurs/compétiteurs spécifiques permettraient de mettre en évidence leur implication, dans différentes phases de traitement.

G) Étude de la cytotoxicité de nanoparticules d'or sur une lignée de cancer du sein triple-négatif

I) Introduction

Les nanoparticules sont de plus en plus développées dans le domaine de la Cancérologie en tant qu'outils de diagnostic ou thérapeutique. En effet, les nanoparticules d'or « nues » présentent l'intérêt d'être biologiquement inertes. Sur celles-ci peuvent être greffés des composés actifs permettant de cibler des protéines d'intérêt (J. Lee et al. 2014). Par ailleurs, elles peuvent présenter des activités cytotoxiques, ou augmenter l'efficacité de la radiothérapie dans le cadre de tumeurs radio-résistantes (Choi et al. 2018). Ainsi, dans le cadre d'une collaboration de recherche entre l'UMR 6533 CNRS/IN2P3, LPC+, Equipe Physico Chimie des Surfaces Nanostructurées et l'UMR INSERM 1240 IMoST, groupe « Résistance », ces travaux avaient pour objectifs (i) la production et la caractérisation de nanoparticules d'or PEYgylées par le laboratoire LPC+ et (ii) l'étude de leur cytotoxicité sur la lignée HCC1937 du cancer du sein triple-négatif. **Ces travaux préliminaires ont fait l'objet d'un article en deuxième auteur publié dans la revue Journal of Biomaterials and Nanobiotechnology (IF = 2,1).**

II) (*Article paru*) Cytotoxicity study of gold nanoparticles on the basal-like triple-negative HCC1937 breast cancer cell line

Cytotoxicity Study of Gold Nanoparticles on the Basal-Like Triple-Negative HCC-1937 Breast Cancer Cell Line

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Abstract

The Triple Negative “Basal-like” breast cancer (TNBL) tumours have a high proliferative capacity and develop a resistance phenotype associated with metastases. However, the management of TNBL carcinomas is still not standardized. Among the promising trails, gold nanoparticles could be a relevant tool for the development of a targeted treatment for this breast cancer subtype in monotherapy, associated and/or conjugated with other drugs. In this work, we report the cytotoxicity impact of gold nanoparticles wrapped in Poly-Ethylene Glycol (PEG) on the TNBL HCC-1937 breast cancer cell line. PEG-coated gold nanoparticles (PEG-Au NPs) were synthesized by a two-step method using a reduction process followed by a post-functionalization called PEGylation. PEG-Au NPs were characterized using transmission electron microscopy and X-ray diffraction. The gold content of the samples was determined using atomic absorption spectrometer. The cytotoxicity tests were performed using Sulforhodamine B survival test and resazurin viability test. PEG-Au NPs impact analysis on HCC1937 TNBL cell line showed a clear toxic action of type dose dependent and at long term. These PEGylated gold nanoparticles present a promising tool for the development of tumor-specific radiosensitizing vectors, with or without the association of other treatment strategies.

Keywords

Breast Cancer, “Basal-Like” Triple Negative, HCC-1937, Gold Nanoparticles, Cytotoxicity

1. Introduction

Gold nanoparticles (GNPs) are promising candidates in the field of medicine for the treatment of illnesses such as Alzheimer's [1], vascular [2], skin [3], sclerosis [4] and cancer [5]. Gold nanoparticles exhibit tunable physical properties making them ideal for developing diagnostic tools [6] [7] [8] [9] or therapeutic treatments using photothermal effect [10]-[15]. Gold nanocomposites can also act as radiosensitizer [16] [17] [18] [19] [20] in order to avoid collateral damages induced by the use of radiotherapy. Gold nanoparticles can also be defined as theranostic tools by combining diagnostic and therapeutic actions [21] [22] [23] [24] [25]. The use of gold nanoparticles to try to cure cancer is a very active field of research. With this nanomedicine strategy, gold nanoparticles are defined to target tumorous cells and minimize side effects [26] [27], to enhance conventional therapies [28] [29] or to develop new diagnostic and therapeutic devices [30] [31]. Despite recent advances in the treatment of cancers, cancer therapy is still a great challenge for the research in nanomedicine. The Triple Negative "Basal-like" breast cancer (TNBL) tumors are particularly aggressive and of poor prognosis. They constitute 70% of the basal-like tumor subtype and 15% of all breast cancers. They are characterized by the non-expression of estrogen receptors (ER) and progesterone receptors (PR), and the absence of HER2 over-expression or ERBB-2 amplification [32] [33] [34]. TNBL tumours have a high proliferative capacity and may respond well to neoadjuvant chemotherapy or develop a resistance phenotype associated with metastases. More, due to their properties, these tumours do not respond to targeted hormonal therapies. Consequently, many targeted therapies using inhibitors of poly (ADP-ribose) polymerase (PARP) or anti-EGFR monoclonal antibodies or combined therapies like anti-EGFR monoclonal antibodies/EGFR tyrosine kinase inhibitors or chemotherapy/radiotherapy are currently in development [35] [36] [37] [38] [39]. However, the management of TNBL carcinomas is still not standardized. Among these promising trails, gold nanoparticles could be a relevant tool for the development of a targeted treatment for this breast cancer subtype in monotherapy, associated and/or conjugated with other drugs [40]. In this context, these works were focused on the cytotoxicity impact of gold nanoparticles wrapped in Poly-Ethylene Glycol (PEG), on the TNBL HCC-1937 breast cancer cell line.

2. Experimental

2.1. Synthesis of PEG-Coated Gold Nanoparticles (PEG-Au NPs)

Before the experiment, all the glassware was carefully cleaned using aqua regia (mixture of concentrated nitric and hydrochloric acid in 1:3 volume ratio) and rinsed with ultra-pure water (18 MΩ.cm), blown dried with nitrogen and stored at 110°C. The starting solution is obtained by dissolving the designated amount of hydrogen tetrachloroaurate (III) hydrate ($\text{HAuCl}_4 \cdot x\text{H}_2\text{O}$) purchased from Sigma Aldrich to reach suitable gold concentration. Gold nanoparticles in suspen-

sion were synthesized using a two step method. First, reduction of hydrogen tetrachloroaurate (III) precursor species was carried out using sodium borohydride under vigorous stirring for 15 minutes at room temperature; gold metal nano-cores were obtained. Secondly, gold nanoparticles surface were post functionalized using thiol-terminated poly(ethylene) glycol (HS-PEG, of MW 5000 from Sigma Aldrich). After this so called PEGylation step, red ruby, stable gold nanoparticles suspensions are obtained.

2.2. Gold Nanoparticles Characterization

2.2.1. Transmission Electron Microscopy (TEM)

A Hitachi transmission electron microscope (H7650) was used for morphological characterization.

2.2.2. X-Ray Diffraction (XRD)

The crystalline structure of the gold nanoparticles was determined using a Bruker D2 Phaser diffractometer. The operating parameters are listed in **Table 1**.

2.2.3. Atomic Absorption Spectroscopy (AAS)

The gold content of the samples was determined using an AA 7000 atomic absorption spectrometer (Shimadzu) with gold hollow-cathode lamp. All analyses were performed at a wavelength of 242.8 nm using 0.7 nm slit width. Before the experiments, the machine was calibrated using a gold standard for AAS (Sigma Aldrich) at 1000 ppm in 5% HCl. Calibration line was performed using thirteen diluted standard solutions prepared in 5 % HCl suitable for trace analysis and high-purity water, 18.2 MΩ, ranging from 0.01 to 20 ppm, respectively.

2.3. Cell Culture

The HCC1937 TNBL breast cancer cell line (ATCC®) was maintained in RPMI 1640 medium (Gibco®), supplemented with 10% decomplexed fetal calf serum and 20 mg/mL gentamycin, in 75 cm² culture dishes (Falcon®) at 37°C under 5% CO₂, according to supplier's instructions [41]. For cytotoxicity tests, cells were detached by trypsinisation, counted with the trypan blue exclusion test, and seeded in 96-well microplates (Falcon®), at a concentration of 2000 per well in 100 µL of medium. Cells were then treated with 100 µL of increasing concentrations of PEG-Au NPs (2, 4, 7, 14 and 25 µM) diluted in the culture medium, for one to seven days. Bright field microscopy observations as well as viability tests (Sulforhodamine B test and resazurin test) of treated and untreated wells allowed the determination of nanoparticles' cytotoxicity.

Table 1. Operating parameters of XRD apparatus.

radiation	tube voltage	tube current	scanning range (2θ) scanning speed	time constant	spinning speed
CuKα1 λ = 1.5406 Å	30 kV	10 mA	30° to 80° 0.24 °/min	5 seconds	15°/min.

2.4. Cytotoxicity Tests

2.4.1. Sulforhodamine B Survival Test

Sulforhodamine B (SRB) is an anionic dye that binds electrostatically to cellular proteins, reflecting indirectly the number of living cells in the wells. Its absorbance peak is at 540 nm. After each treatment period (from Day 1 to Day 7) cells were fixed with 50 μ L of a 50% (weight/volume) trichloroacetic acid (TCA) solution added to each well for 1 hour at 4°C. Plates were then washed, dried, and 50 μ L of a 0.4% (w/v) solution of SRB in 1% (v/v) acetic acid was added to each well. After 15 minutes of incubation at room temperature, plates were washed with 1% acetic acid solution and dried. 200 μ L of a 10 mM tris-base buffer solution was finally added to the wells for dye solubilisation and plates were stirred for 30 min. The optical density (OD) of each well was determined at 540 nm using a microplate reader (VWR Multiskan FC®). Cell survival ratio was calculated by the following formula: cell survival (%) = (Mean (OD $\lambda_{540\text{ nm}}$) of treated wells / Mean (OD $\lambda_{540\text{ nm}}$) of untreated control wells) $\times$ 100.

2.4.2. Resazurin Viability Test

Resazurin test is a colorimetric metabolic assay based on the reduction of resazurin into resorufin by metabolically active cells. Resorufin is a fluorescent dye exhibiting a pink colouring. After a time of incubation with resazurin, the amount of resorufin formed is proportional to the number of living cells in the wells. For these experiments, after each treatment period (from Day 1 to Day 7), medium was removed from the plates and cells were incubated with 100 μ L of 60 μ M resazurin diluted in PBS*, for 4 hours. Optical density (OD) of resorufin and resazurin were measured at 570 and 620 nm, respectively with a microplate reader (VWR Multiskan FC®). The corrected OD of resorufin was calculated as follows: (OD resorufin) = ((OD) $\lambda_{570\text{ nm}}$ - (OD) $\lambda_{620\text{ nm}}$). Then, the percentage of cell survival was obtained with the following formula: Cell survival (%) = (Mean (OD resorufin) of treated wells / Mean (OD resorufin) of untreated control wells) $\times$ 100.

The half maximal inhibitory concentration (IC50) was calculated with the following formula, $IC_{50} = \text{EXP}(\text{LN}(\text{concentration} > 50\% \text{ inhibition}) - ((\text{signal} > 50\% \text{ inhibition} - 50) / (\text{signal} > 50\% \text{ inhibition} - \text{signal} < 50\% \text{ inhibition})) * \text{LN}(\text{concentration} > 50\% \text{ inhibition} / \text{concentration} < 50\% \text{ inhibition}))$ (1) [42].

2.5. Statistical Analysis

Cytotoxicity results were expressed as means $\pm$ standard error of n independent experiments. All experiments were performed at least in triplicate and then statistically compared using a Student's t-test. Tests were two-sided and the nominal level of significance was $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****) and $p < 0.00001$ (*****).

3. Results and Discussion

3.1. Gold Nanoparticles in Suspension

The TEM images of the synthesized gold nanoparticles in suspension are shown

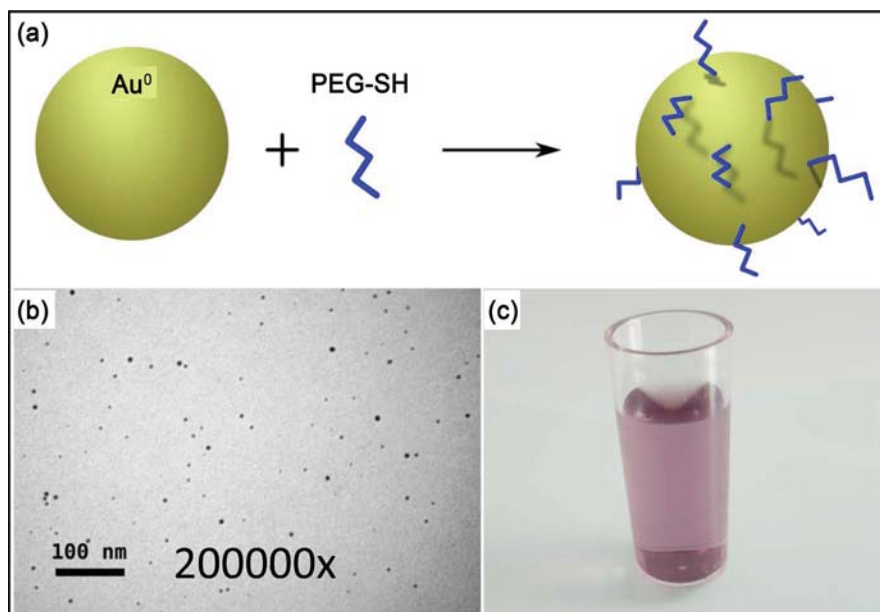


Figure 1. (a) Functionalization of Au NPs with PEG-SH; (b) High transmission electron microscopy images of PEG-coated gold nanoparticles suspensions (PEG-Au NPs); (c) Photo of PEG-coated gold nanoparticles suspensions (PEG-Au NPs) in the beaker.

in **Figure 1**.

(PEG-Au NPs) pictures exhibited a quasi-spherical shape, with some aggregates. Taking into account this spherical geometry, the average core diameters of PEG-coated gold nanoparticles are found to be 7 nm. PEG-coated gold nanoparticles are synthesized using sodium borohydride as a strong reducing reagent. The PEG-coating of the gold nanoparticles, also known as pegylation, is dedicated to improve the residence in body [43] and to protect the nanoparticles from the immune system [44].

3.2. X-Ray Diffraction Analysis

XRD pattern of the analyzed gold nanopowder is presented in **Figure 2**. Intense peaks, relative to Bragg's reflections, could be indexed considering the face-centered cubic gold structure. No diffractions due to crystallographic impurities were found. A strong diffraction peak located at 38.7° was ascribed to the (111) facets of the gold face-centered cubic structure. The other diffraction peaks relative to (200) (220) and (311) facets were much weaker. The broadening of Bragg's peaks indicates the formation of nanoparticles. The mean size of gold nanoparticles was calculated using the Debye Scherrer's equation by determining the width of the (111) Bragg reflection. The crystallite size, D , is evaluated by measuring the width of the curves produced and using the Scherrer Formula (2):

$$D = \frac{0.9 \cdot \lambda}{\beta \cos \theta} \quad (2)$$

where λ is the wavelength of the Cu K α 1 line in Å, β is the full-width at half max (FWHM) of the peak. The size of the crystallites was found and is approximately

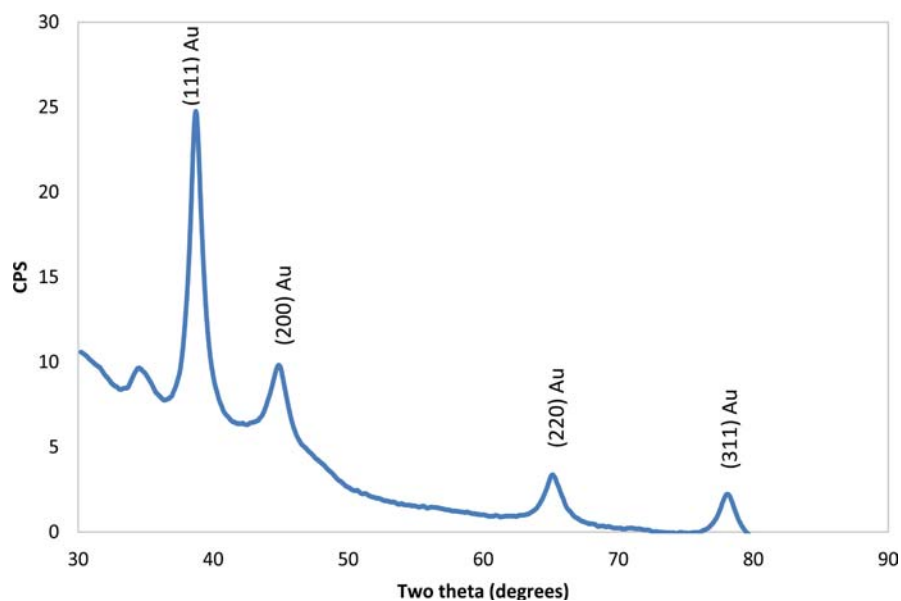


Figure 2. XRD spectrum of the gold nanopowder, the principal Bragg reflections are indexed.

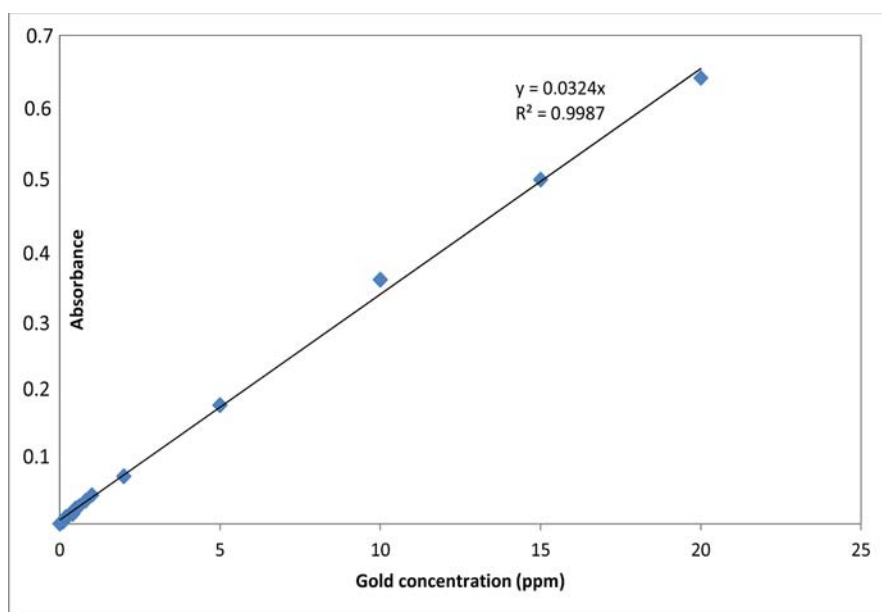


Figure 3. Calibration line was obtained by assaying thirteen diluted standard solutions ranging from 0.01 to 20 ppm, respectively.

8 nm for gold nanopowder. This result is in good agreement with the average core diameter of 7 nm previously found.

3.3. Atomic Absorption Spectroscopy (AAS)

The calibration line for gold assay is presented in **Figure 3**. This calibration line was made by assaying thirteen diluted standard solutions ranging from 0.01 to 20 ppm, respectively. The calibration coefficient (r^2) obtained from the calibration line was 0.9987.

We measured the gold nanoparticles suspensions using the 5% HCl solution to dilute the suspension. The gold concentration in our samples is 19.5 ppm.

3.4. PEG-Au NPs Cytotoxicity Analysis on HCC1937 TNBL Cell Line

The action of increasing concentrations of PEG-Au nanoparticles (2 to 25 μM) was studied on the Triple-Negative Basal-Like breast cancer cell line HCC1937, during seven days. For this, i) SRB cell survival test coupled to cell microscopy analyses and ii) cell viability *via* resazurin test were performed.

The rate of HCC1937 TNBL cell viability *via* Sulforhodamine B quantitative survival test was performed in order to quantify PEG-Au NPs toxicity on this breast cancer cell line. For this, after each treatment period, the absorbance of the Sulforhodamine B dye fixed in each well was quantified and allowed the calculation of the percentage of cell survival compared to untreated cells presented in **Figure 4**.

Control cells treated with PEG alone showed no difference in cell survival compared to untreated cells (*data not shown*). After two days of treatment with 2 and 4 μM of nanoparticles, cell survival was respectively of $108\% \pm 2\%$ and $109\% \pm 2\%$. Then, after 5 days, it decreased to $82\% \pm 1\%$ with 2 μM and to $61\% \pm 2\%$ with 4 μM ($p < 0.00001$ and $p < 0.00001$ respectively), and remained stable until 7 days with $85\% \pm 1\%$ with 2 μM , ($p = 0.80$) and $59\% \pm 3\%$ with 4 μM ($p = 0.25$). Cell survival ratio of HCC1937 cell line treated with 7 μM nanoparticles was of $98\% \pm 2\%$ after one day of treatment, and decreased significantly to $39\% \pm 1\%$ ($p < 0.00001$) and $28\% \pm 2\%$ ($p < 0.00001$) after 5 and 7 days of treatment, respectively. Then, with higher nanoparticles concentrations (14 and 25 μM), cell survival was of $96\% \pm 1\%$ and $89\% \pm 1\%$ after one day of treatment, and

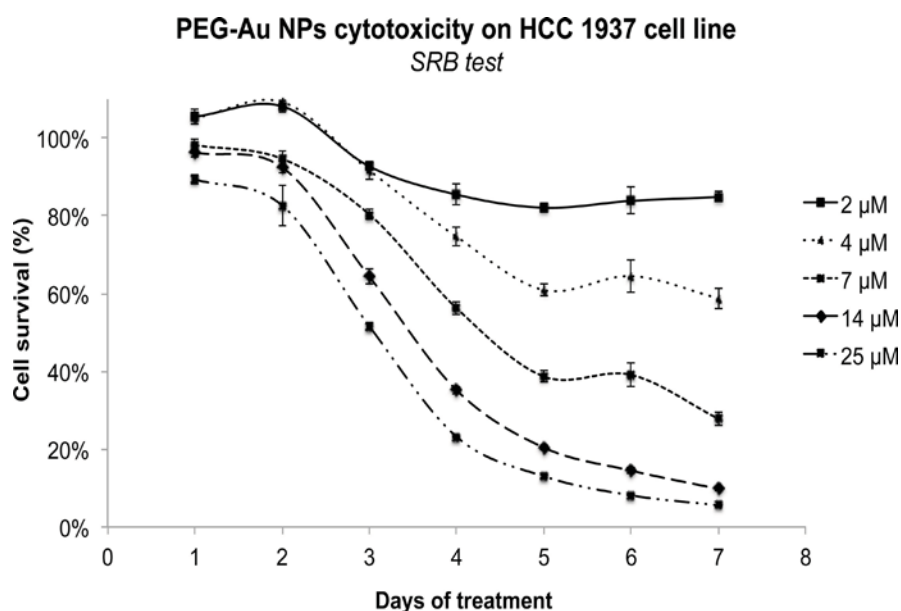


Figure 4. Mean percentage of HCC1937 cells viability determined with the Sulforhodamine B test after 1 to 7 days of treatment with increasing concentrations of PEG-Au NPs, (**** = $p < 0.00001$, bars on plots = standard errors).

dropped dramatically to $20\% \pm 0.7\%$ ($p < 0.00001$) and $13\% \pm 0.4\%$ ($p < 0.00001$) after 5 days of treatment. Then, it continued to decrease until 7 days of treatment, with survival rates of $10\% \pm 0.4\%$ ($p < 0.00001$) and $6\% \pm 0.2\%$ ($p < 0.00001$) for 14 and 25 μM of nanoparticles, respectively. All these results showed that PEG-Au NPs toxicity on HCC1937 cell line was both dose and time-dependent. In addition, microscopy analyses presented in **Figure 5** showed clearly detached cells after treatment with 25 μM PEG-Au nanoparticles for seven days (**Figure 5(b)**) compared to controls (**Figure 5(a)**), in which cells have reached the confluence.

Then, the resazurin viability test involving the reduction of resazurin into resorufin by metabolically active cells was performed. The percentages of cell viability after treatment with increasing concentrations of PEG-Au NPs were calculated by the ratio of metabolic activity of untreated cells, after 1 to 7 days of treatment, see **Figure 6**. Similarly to SRB test, control cells with PEG alone were

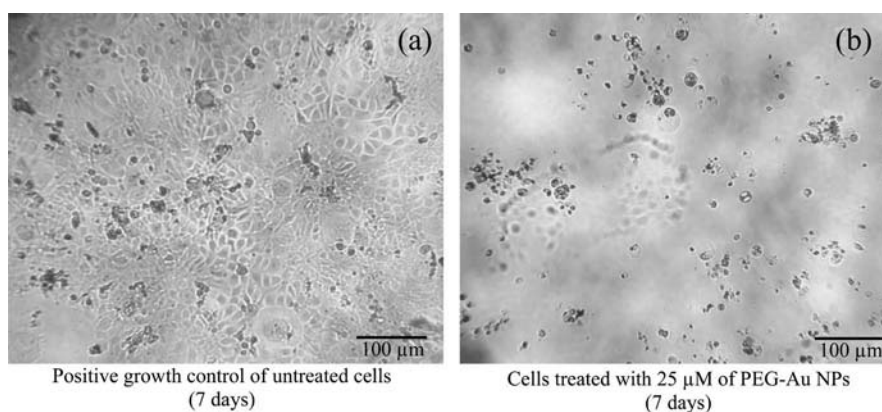


Figure 5. Bright field observations of HCC1937 cells after seven days of incubation (a) without treatment and (b) after treatment with 25 μM PEG-Au NPs.

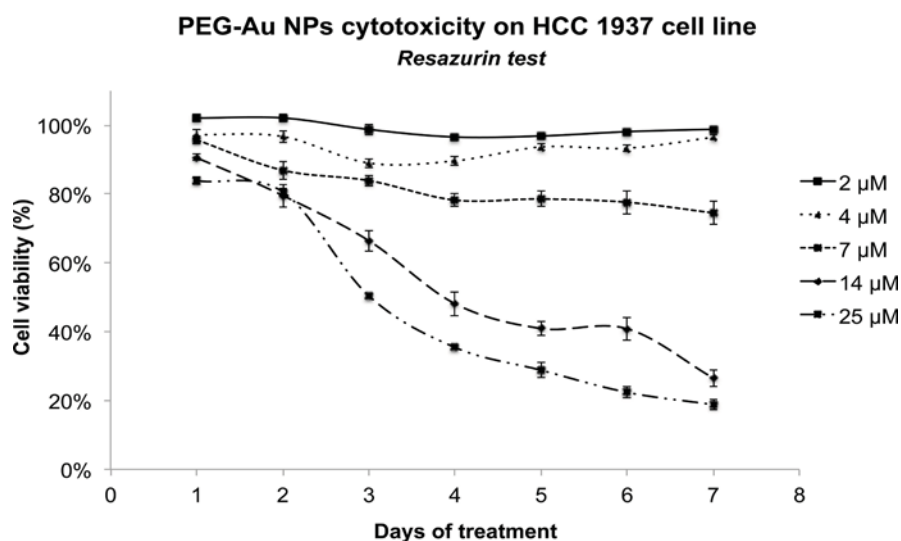


Figure 6. Mean percentage of viability of HCC1937 cells treated for 1 to 7 days with increasing concentrations of PEG-Au NPs (resazurin test), (bars on plots = standard errors).

performed, and toxicity was not noted compared to untreated cells (*Data is not shown*). Then, after a treatment with 2 μM of nanoparticles, cell viability was of $102\% \pm 1\%$ at day 1, and remained stable until the end of the treatment with $99\% \pm 1\%$ at day 7 ($p = 0.28$). A slight decrease in cell viability was detected after 3 days of treatment with 4 μM ($92\% \pm 1\%$, $p < 0.01$), which remained stable until seven days ($96\% \pm 1\%$, $p = 0.79$). After a treatment with 7 μM of nanoparticles, cell viability was of $96\% \pm 1\%$ after one day, decreased significantly to $78\% \pm 2\%$ ($p < 0.00001$) after 4 days, and remained stable until the seventh day ($75\% \pm 3\%$, $p = 0.54$). In contrast, with a higher nanoparticles concentration of 14 μM , cell viability dropped from $91\% \pm 1\%$ after one day to $48\% \pm 3\%$ after 4 days ($p < 0.00001$), remained relatively stable until the sixth day ($41\% \pm 3\%$, $p = 0.09$) and decreased at day 7 ($27\% \pm 2\%$, $p < 0.01$). Finally, the highest nanoparticles concentration of 25 μM induced a dramatic decrease in cell viability from $84\% \pm 1\%$ at day 1, to $35\% \pm 1\%$ at day 4 ($p < 0.00001$), and to $19\% \pm 2\%$ at day 7 ($p < 0.00001$). Similarly to SRB results, these results showed a clear dose and time-dependent toxicity of PEG-Au NPs nanoparticles on HCC1937 breast cancer cell line.

4. Discussion

PEG-Au NPs impact analysis on HCC1937 TNBL cell line showed a clear toxic action of type dose dependent and at long term. Indeed, TNBL cells mortality increased by the time, with IC_{50} (SRB test) of 9 μM at day 4 and of 5 μM at day 7. Similarly, IC_{50} (Resazurin test) was of 12 μM at day 4 and decreased to 10 μM at day 7. The variability between SRB survival test and resazurin viability test can be explained by a difference in sensibility thresholds due to the inner mechanism of both tests. Nevertheless, these two assays described the same toxicity profile of PEG-Au NPs nanoparticles on HCC1937 cell line, probably correlated with nanoparticles accumulation in cells.

Similarly, in previous *in vitro* and *in vivo* studies, gold nanoparticles have been used as tool both to detect and to treat breast cancer [45]. Indeed, the mostly used strategies were the ones coupling the hyperthermia effect with selective functionalization systems to optimize the cytotoxicity [46]. In general, gold nanoparticles cytotoxicity effect is mainly related to gold nanoparticle's concentration, time of exposure, and nanoparticle's size. Therefore, it would be relevant to analyse these different parameters on models of breast cancer cell cultures that mimic more faithfully the shape of tumors as 3D cell culture. Indeed, in comparison to monolayer cell culture, a systematic lowered sensitivity was detected for 3D cell culture in response to various cytotoxic drugs, probably due to 3D cellular heterogeneity and/or resistance phenotypes [47]. These studies are currently underway.

5. Conclusion

PEG-Au NPs were synthesized using a two-step method using a reduction process followed by a post-functionalization called PEGylation. Stable gold nanoparticles in suspension, suitable for biological experiments, with a gold concentration of

19.5 ppm, are obtained. The two cytotoxicity tests described the same PEG-Au NPs nanoparticles toxicity profile of type dose and time dependent on HCC1937 cell line, probably correlated with nanoparticles accumulation in cells. These works open the way to further experiments aiming to determine the PEG Au NPs uptake and intracellular localisation in TNBL cell line models.

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III) Conclusion sur les travaux

Ces premiers travaux préliminaires ont permis de mettre en évidence la cytotoxicité de ces nanoparticules d'or PEGylées sur la lignée **HCC1937**. Des études complémentaires sont nécessaires afin de caractériser la fixation et la nature de toxicité de ces nanoparticules.

Conclusion générale Et perspectives

Les travaux de recherche réalisés au cours de ma thèse avaient pour objectif le développement d'outils diagnostics permettant de modéliser *in vitro* l'optimisation du traitement des tumeurs mammaires Triple-Négatives (TN). Pour ce faire, la première partie de ces travaux s'est axée sur le développement des modèles de cultures cellulaires plus prédictifs, notamment des cultures cellulaires en trois dimensions, à partir de différentes lignées de cancer du sein TN. En effet, les sphéroïdes miment plus fidèlement la conformation des tumeurs et permettent une analyse *in vitro* plus fine de l'impact de la cytotoxicité/résistance des chimiothérapeutiques. A partir de ces modèles de tumeurs très agressives et de mauvais pronostic, ces travaux ont consisté au choix de la technique de culture 3D la plus adaptée, à l'identification des paramètres essentiels permettant ce développement, ainsi qu'à la mise en place et à l'adaptation des outils permettant l'analyse et la caractérisation des sphéroïdes. Particulièrement, ces travaux ont mis en évidence deux profils de sphéroïdes différents, un profil prolifératif pour la lignée MDA-MB-231 et un profil non-prolifératif pour la lignée SUM1315. En outre, les molécules fortement cytotoxiques peuvent être mises en évidence à partir de modèles prolifératifs comme non prolifératifs. Cependant, les molécules cytostatiques ne peuvent être identifiées qu'à partir de modèles présentant une prolifération. Ainsi, la détermination de ces profils de sphéroïdes apparaît comme une étape primordiale. En effet, elle permet une utilisation optimale de ces modèles dans les tests de screening et d'analyse de l'efficacité des médicaments, dans le but de prédire au mieux leur mécanisme d'action (**Dubois et al., 2017**). Le développement des cultures en 3D a également été réalisé, dans les mêmes conditions expérimentales, à partir de trois autres lignées issues de tumeurs mammaires triple-négatives, HCC1937, SW527 et DU4475. Ces trois nouveaux modèles présentaient également un profil prolifératif similaire à la lignée MDA-MB-231. Parallèlement, afin de normaliser et ainsi augmenter la reproductibilité des expériences réalisées avec les modèles 3D, un milieu synthétique (sans sérum de veau ajouté), intitulé **OptiPASS** a été adapté à ces sphéroïdes. Ce travail, initié en collaboration avec des industriels de la société BIOPASS, a permis de déterminer la valeur ajoutée de ce milieu, en termes (1) de facilité de production des sphéroïdes en utilisant la même méthodologie précédemment développée, (2) de rétablissement du profil prolifératif des sphéroïdes issus de la lignée SUM1315 mise en culture dans ce milieu, (4) de sensibilité favorable de ces lignées 3D vis-à-vis des molécules chimiothérapeutiques et (3) de vitesse de prolifération des sphéroïdes. Le milieu de culture

OPTIPASS semble être totalement adapté aux cultures en 3D des lignées cellulaires TN et d'autres modèles sont actuellement en cours de développement.

Dans un deuxième temps, les modèles de sphéroïdes TN ainsi développés ont été utilisés pour déterminer l'efficacité d'un co-traitement utilisant l'anti-PARP1 Olaparib en combinaison avec la radiothérapie fractionnée. Les tests de survie globale et de survie clonogénique réalisés dans un premier temps sur les lignées cellulaires SUM1315 et MDA-MB-231 en 2D ont permis de mettre en évidence une forte toxicité du co-traitement en comparaison avec un traitement seul, par radiothérapie ou avec Olaparib. Par ailleurs, les analyses ANOVA effectuées à partir des données de survie ont permis de définir cette toxicité comme étant dépendante (1) de la dose de radiothérapie et (2) de la durée d'exposition à l'Olaparib. Également, (3) cet effet potentialisateur du co-traitement était effectif en présence de doses relativement faibles d'Olaparib. Ainsi, afin de mimer plus précisément la réponse prédictive des cellules tumorales à ce co-traitement, des expériences similaires ont été réalisées sur des sphéroïdes. En effet, afin de confirmer ces résultats en termes de doses efficaces, les expériences sur les cultures 3D ont été réalisées en présence d'une forte dose (50 μ M) et d'une faible dose (5 μ M) d'Olaparib et à plus long terme (6, 8 ou 10 jours), avec le même fractionnement de radiothérapie. Ce traitement à long terme a été rendu possible à partir des cultures en 3D, capables d'être maintenues en culture dans des conditions biomimétiques pendant 14 jours, à contrario des cultures en 2D. Ces analyses ont mis en évidence une forte inhibition de croissance et l'induction de la mortalité des cellules formant les sphéroïdes suite au co-traitement. De la même manière, les analyses ANOVA réalisées à partir des données de viabilité ont mis en évidence une absence de bénéfice à traiter les sphéroïdes avec la forte dose d'Olaparib de 50 μ M, en comparaison de la dose de 5 μ M. Ainsi, ces résultats ont permis de confirmer le potentiel intérêt clinique d'une administration de l'Olaparib au long cours, à faible dose, et ce pendant et après les séances de radiothérapie. Par ailleurs, dans cette étude de co-traitement avec l'anti-PARP1 et la radiothérapie fractionnée, une hétérogénéité de réponse entre les deux lignées cellulaires étudiées (MDA-MB-231 et SUM1315) a été mise en évidence. Ainsi, les études transcriptomiques, réalisées à partir des données de la littérature, ont permis d'identifier des profils génétiques très différents entre ces deux lignées très agressives de tumeurs mammaires. Particulièrement, la

lignée SUM1315 serait issue d'un foyer de cellules métastatiques hautement indifférenciées et possiblement engagées dans la lignée neuronale. Cette piste pourrait être explorée avant d'envisager un transfert clinique de cette méthodologie de co-traitement. En effet, différents essais cliniques (10) sont en cours de recrutement afin de définir la dose d'Olaparib maximale tolérée en combinaison avec la radiothérapie sur des patients atteints de cancers tête et cou, de cancers du poumon, de cancers du sein ou encore de **gliomes**. (« **NCT03212742 - Phase I/IIa Study of Concomitant Radiotherapy With Olaparib and Temozolomide in Unresectable High Grade Gliomas Patients** » s. d.). (« **NCT03109080 - Olaparib & Radiation Therapy for Patients Triple Negative Breast Cancer (TNBC)** » s. d.) (« **NCT01562210 - Olaparib Dose Escalating Trial + Concurrent RT With or Without Cisplatin in Locally Advanced NSCLC** » s. d.) (« **NCT02229656 - Olaparib and Radiotherapy in Head and Neck Cancer** » s. d.). Dans ce contexte, ces travaux pourraient ouvrir la perspective clinique d'un co-traitement des métastases cérébrales de tumeurs mammaires triple-négatives, très fréquentes chez ce sous-type tumoral. Le transfert des résultats de ce co-traitement vers les modèles *in vivo* serait envisageable, après avoir analysé son efficacité à plus long terme, sur des modèles de métastases cérébrales issues de tumeurs TN.

Par la suite, afin de mieux caractériser la réponse de ces modèles de tumeurs TN suite au traitement par Olaparib, mes travaux de thèse ont porté sur le développement d'un outils diagnostique dérivé de l'Olaparib et couplé à un fluorochrome (Ola-FL). Ce dernier a été utilisé comme un outil permettant de suivre le degré de pénétration et la distribution de cet anti-PARP, dans des modèles de sphéroïdes TN. Le marqueur fluorescent Ola-FL a dans un premier temps été caractérisé à partir de cultures 2D par imagerie en fluorescence. Ces travaux ont permis de confirmer la rapide pénétration et la fixation spécifique de l'Ola-FL sur sa cible cellulaire, et pour les trois lignées étudiées (MDA-MB-231, SUM1315 et HCC1937). Ensuite, l'analyse de la pénétration et de la distribution de l'Ola-FL au sein des sphéroïdes après 4h d'incubation a permis de démontrer (1) une pénétration rapide de l'Ola-FL dans l'intégralité des sphéroïdes (inférieure à 5 min), (2) une fixation de l'Ola-FL dans les noyaux des cellules formant le sphéroïde, ainsi (3) qu'une persistance de ce dernier dans tous les sphéroïdes après 3h d'incubation, et ce même dans les zones hypoxiques/quiescentes des sphéroïdes présentant des gradients d'hétérogénéité cellulaire (sphéroïdes âgés de plus de 10 jours).

L'ensemble de ces travaux laissent supposer que l'Ola-FL semble être un outil de diagnostic efficace pour analyser le degré de pénétration/répartition/maintien de cet anti-PARP dans les sphéroïdes, afin de prédire son degré d'efficacité dans différents modèles de tumeurs TN.

Dans un deuxième temps, afin d'étudier le maintien/l'efficacité de l'Olaparib dans les modèles de tumeurs TN, l'analyse de la co-expression de deux pompes *Multi Drug Resistance* d'intérêt clinique, la MRP7 et la P-gp, a été effectuée en réponse au traitement par Olaparib. Ces travaux ont été réalisés sur les lignées cellulaires MDA-MB-231 et SUM1315 en condition 2D et 3D. En effet, l'analyse de la co-expression des pompes MRP7 et P-gp a été réalisée sur des modèles de culture 2D à court-terme, après 4h de traitement avec 5 ou 50 μ M d'Olaparib. Par contre sur des modèles de culture 3D, ces études ont été effectuées à plus long-terme, après 8 à 10 jours de traitement avec une faible dose d'Olaparib de 5 μ M, reflétant ainsi la résistance associée à ces pompes d'efflux dans les cellules résiduelles persistantes, après le traitement. Tout d'abord, sur les cultures 2D, une expression de type relai de la MRP7 et de la P-gp a été mise en évidence sur le modèle MDA-MB-231 et ce pour les deux doses d'Olaparib testées. En effet, une induction d'expression de la P-gp au dépend de la MRP7 a été détectée en présence de l'Olaparib. Pour la lignée SUM1315, aucune induction de la pompe MRP7 n'a été identifiée en présence de ces deux concentrations d'anti-PARP. Cependant, nos études antérieures avaient déjà démontré une co-expression et une induction de type relai de la BCRP (une autre pompe MDR majeure) et de la P-gp, chez la lignée SUM1315 après le même temps d'incubation et en présence de 50 μ M d'Olaparib. L'engagement de la BCRP et de la P-gp dans la résistance à l'Olaparib dans les cellules SUM1315 selon ces conditions expérimentales pourrait expliquer l'absence d'induction de MRP7. Par la suite, l'analyse de l'expression des pompes MRP7 et P-gp effectuée sur des coupes de sphéroïdes traités à long terme (10 jours) avec 5 μ M d'Olaparib ont mis en évidence une nette surexpression de la P-gp dans cellules des sphéroïdes MDA-MB-231 et SUM1315 résiduelles persistantes, après le traitement avec l'Olaparib. Ces résultats montrent non seulement l'expression de type relai de différentes pompes MDR majeures dans les deux lignées TN en 2D et 3D, lors de traitement avec l'Olaparib mais également, le rôle relevant de la P-gp face à ce traitement. Des expériences d'analyses de l'activité de ces MDR en présence d'inhibiteurs/compétiteurs spécifiques de chacune des pompes permettrait de mettre en évidence leur implication dans différentes phases de traitement. Par ailleurs, les coupes de sphéroïdes traités par l'Olaparib présentaient

systematiquement des zones creuses situées au centre des sphéroïdes, en comparaison avec des sphéroïdes contrôles. L'absence de cellules au centre des sphéroïdes pourraient refléter une efficacité plus importante de l'Olaparib sur les cellules situées au centre des sphéroïdes.

Ainsi, l'ensemble des analyses de pénétration et de distribution de l'Ola-FL, dérivé de l'Olaparib et la co-expression des pompes MRP7 et P-gp au sein des sphéroïdes traités par cet anti-PARP témoignerait de l'efficacité de l'Olaparib à faible dose, notamment dans les zones hypoxiques des sphéroïdes. La persistance des cellules résistantes résiduelles dans des zones périphériques des sphéroïdes après le traitement avec cette faible dose d'Olaparib pourraient être en relation avec l'activité des pompes d'efflux MDR.

L'ensemble de ces travaux de thèse ont permis d'initier, depuis 2017, une collaboration de type public/privé avec deux partenaires industriels, les sociétés **BIOPASS** et **BIOMARQUEURS**, spécialisées dans le domaine des Biotechnologies pour la santé. Suite à cette collaboration le projet **BIORCELL3D**, doté d'un financement des fonds européens FEDER a été mise en place depuis septembre 2017. Celui-ci, d'une durée estimée de trois ans a pour objectifs (i) le développement puis la commercialisation à grande échelle des modèles cellulaires 3D tumoraux ou sains et (ii) la mise en place d'une plateforme de screening *in vitro* permettant d'évaluer l'efficacité des molécules en développement. Ces perspectives industrielles incluent (1) le développement et la caractérisation des modèles 3D en co-cultures (avec des cellules fibroblastiques, des cellules immunitaire ou endothéliales) permettant d'obtenir des modèles encore plus prédictifs de la réponse tumorale *in vivo*, et (2) la généralisation du milieu OptiPASS pour la formation des cultures cellulaires 3D, permettant ainsi une optimisation de la reproductibilité des études de screening réalisées à partir de ces modèles. Parallèlement au projet **BIORCELL3D**, un projet de développement d'outils compagnons utilisant des molécules marquées fluorescentes pour le diagnostic et l'évaluation de la réponse tumorale est en cours (brevet LightSpot en cours de rédaction). Dans ce contexte, à la suite de cette mission de Recherche Doctorale, j'aurai l'opportunité de participer à ce projet de transfert technologique en intégrant le consortium **IMoST/BIOMARQUEURS/BIOPASS** dans un laboratoire en commun (projet de LABCOM).

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Résumé

Le cancer du sein est une maladie complexe et difficile à caractériser. Parmi les différents sous-types moléculaires, les tumeurs du sein Triple-Négatives (TN) sont particulièrement agressives et de mauvais pronostic. Elles sont caractérisées par une absence d'expression des récepteurs aux œstrogènes (ER), à la progestérone (PR), l'absence de surexpression du récepteur *Human Epidermal growth factor 2* (HER2) et de fréquentes mutations sur les gènes BRCA1/2 (profil « BRCAness »). En absence de thérapies ciblées efficaces, de nombreux traitements ciblés notamment les inhibiteurs de poly-ADP-ribose polymérases (anti-PARPs) sont actuellement en cours de développement, en recherche préclinique et clinique. Basés sur le principe de létalité synthétique, les anti-PARPs ciblent les propriétés *BRCAness* des tumeurs TN. Dans ce contexte, ces travaux de recherche ont été orientés sur le développement d'outils diagnostics afin d'optimiser l'efficacité des anti-PARPs sur des tumeurs TN. Pour ce faire, dans un premier temps, des cultures cellulaires en 3D *via* la technique *Liquid Overlay* ainsi que des tests de cytotoxicités associés ont été développés, à partir des lignées cellulaires MDA-MB-231 et SUM1315 de phénotype TN. Ces deux modèles de sphéroïdes ont ensuite été optimisés/normalisés dans un milieu de culture synthétique intitulé OPTIPASS (BIOPASS). Dans un deuxième temps, l'efficacité d'un co-traitement combinant l'anti-PARP1 Olaparib à faibles et à fortes doses et la radiothérapie fractionnée (5x2 Gy) a été modélisée sur les deux lignées MDA-MB-231 et SUM1315, en conditions 2D et 3D. Ces expériences ont clairement mis en évidence un effet potentialisateur de l'Olaparib sur la radiothérapie (i) en présence de faibles doses de cet anti-PARP (5 μ M ou inférieur) (ii) à long terme et (iii) en présence d'un fractionnement maximum (5x2 Gy). De plus, les lignées tumorales TN étudiées présentaient des différences de sensibilité vis-à-vis du co-traitement. Ainsi, une analyse transcriptomique *in silico* a mis en évidence des profils très différents de ces lignées hautement métastatiques et très agressives. Notamment, la lignée SUM1315 semblait présenter un engagement neuronal, suggérant son origine métastatique cérébrale. Ces résultats encourageants pourraient ouvrir de nouvelles perspectives pour le traitement des métastases cérébrales de tumeurs mammaires TN, très fréquentes chez ce sous-type. Dans un troisième temps, afin de mieux caractériser le mode d'action de l'Olaparib sur ces modèles de sphéroïdes, un dérivé fluorescent de l'Olaparib, l'Ola-FL, a été synthétisé et caractérisé. L'analyse de la pénétration et de la distribution de l'Ola-FL au sein des sphéroïdes MDA-MB-231 et SUM1315 a mis en évidence une distribution rapide et homogène du composé ainsi que sa persistance après 3h d'incubation, dans toute la profondeur des sphéroïdes et notamment dans les zones hypoxiques centrales. Enfin, l'analyse de la co-expression de deux pompes *Multidrug Resistance* (MDR) majeures, la MRP7 et la P-gp après le traitement des deux lignées TN avec l'Olaparib, a mis en évidence sur les cultures 2D, une expression de type relai de la MRP7 et la P-gp. Sur les sphéroïdes traités avec une faible dose d'Olaparib à long terme, une expression basale de la MRP7 et une surexpression de la P-gp ont été détectées, au sein des cellules résiduelles périphériques des sphéroïdes. Ces résultats mettent clairement en évidence l'implication des pompes d'efflux dans les mécanismes de résistances à l'Olaparib, dans ces tumeurs agressives.

L'ensemble des résultats issus de la modélisation de l'action de l'Olaparib sur des sphéroïdes MDA-MB-231 et SUM1315 laissent supposer sa plus grande efficacité à faible dose et à long-terme, notamment dans les zones hypoxiques des sphéroïdes, probablement aussi à l'origine de son effet potentialisateur avec la radiothérapie.

Mots clés : Cancer du sein Triple-Négatif CSTN, culture 3D sphéroïdes, milieu de culture sans sérum, anti-PARP1 Olaparib, radiothérapie fractionnée combinée, MultiDrug Resistance, MRP7, P-gp, Ola-FL

Abstract

Breast cancer is a very complex and heterogeneous disease. Among the different molecular subtypes, Triple-Negative (TN) breast cancers are particularly aggressive and of poor prognosis. TN tumours are characterized by a lack of estrogen receptors expression (ER), progesterone receptors expression (PR), the absence of Human Epidermal growth factor receptor 2 overexpression (HER2) of the frequent mutations on BRCA1 / 2 genes ("BRCAness" phenotype). In the absence of effective targeted therapies, many targeted therapies including poly-ADP-ribose polymerase inhibitors (anti-PARPs) are currently under development in preclinical and clinical studies. Based on the synthetic lethality concept, the anti-PARPs specifically target the *BRCAness* properties of TN tumors. In this context, these works were focused on the development of diagnostic tools for the optimization of TN tumours treatment with anti-PARPs. For this, firstly, 3D cell cultures formed with the *Liquid Overlay* technique as well as associated cytotoxicity tests were developed, from the TN breast cancer cell lines MDA-MB-231 and SUM1315. These two spheroid models were then optimized and standardized in a synthetic culture medium called OPTIPASS (BIOPASS). Secondly, the efficacy of a co-treatment combining anti-PARP1 Olaparib at low and high doses and fractionated radiotherapy (5x2 Gy) was analyzed on the two cell lines MDA-MB-231 and SUM1315 cultured in 2D and 3D conditions. These experiments clearly demonstrated a potentiating effect of Olaparib on radiotherapy (i) in presence of low doses of this anti-PARP (5 μ M or inferior) (ii) at long term and (iii) in presence of the maximum fractionation (5x2 Gy). In addition, these two TN cell lines showed a heterogeneous sensitivity to the co-treatment. Thus, an *in silico* transcriptomic analysis revealed very different profiles of these highly metastatic and highly aggressive cell lines. Notably, the SUM1315 cell line presented a neuronal commitment, suggesting its cerebral metastatic origin. These promising results could open up new perspectives for the treatment of TN tumours brain metastases, which are very common in this subtype. Thirdly, in order to better characterize the mode of action of Olaparib on these spheroid models, a fluorescent derivative of Olaparib, Ola-FL, was synthesized and characterized. The analysis of Ola-FL penetration and distribution in MDA-MB-231 and SUM1315 spheroids showed a rapid and homogeneous distribution of the compound as well as its persistence after 3h of incubation, in all the depth of the spheroids and especially in the central hypoxic zones. Finally, the analysis of the co-expression of two major Multidrug Resistance (MDR) pumps, MRP7 and P-gp after the treatment of the two TN lines with Olaparib, revealed on 2D cultures, a relay type expression of the MRP7 and the P-gp. On spheroids treated with a low dose of Olaparib at long term (10 days), a basal expression of MRP7 and an overexpression of P-gp were detected in the peripheral residual cells of the spheroids. These results clearly highlighted the involvement of these efflux pumps in Olaparib resistance mechanisms, in these aggressive tumors.

All the results resulting from the modeling of the action of Olaparib on MDA-MB-231 and SUM1315 spheroids suggest its greater efficacy at low dose and at long-term, especially in the hypoxic zones of the spheroids. This parameter might be probably at the origin of its potentiating effect with radiotherapy.

Keywords: Triple-Negative Breast Cancer TNBC, 3D cell culture, spheroids, serum-free culture medium, anti-PARP1 Olaparib, combined fractionated radiotherapy, Multidrug Resistance, MRP7, P-gp, Ola-FL