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Etude du potentiel des molécules phytochimiques comme adjuvants thérapeutiques dans le cancer du sein

Thu Ha Pham

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Par

Thu Ha PHAM

Etude du potentiel des molécules phytochimiques comme adjuvants thérapeutiques dans le cancer du sein

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ABRÉVIATIONS

4E-BP1	eIF4E binding protein-1
AF-1	Activation function 1
AF-2	Activation function 2
AhR	Aryl hydrocarbon receptor
AHRR	Aryl hydrocarbon receptor repressor
Akt /PKB	PAC-alpha serine/threonine-protein kinase/ Protein kinase B
AIB1/SRC3	Amplified-in-breast-cancer-1
AIP	Aryl hydrocarbon receptor-interacting protein
Api	Apigénine
ARNT	Aryl hydrocarbon receptor nuclear translocator
AUC	Aire sous la courbe
BCRP	Protéine de résistance au cancer du sein
bHLH	Basic helix-loop-helix
CD	Catalytic domain
CDK	Cyclin Dependant Kinase
CO	Contraceptifs oraux
CS	Cancer du sein
DBD	DNA Binding Domain
DES	Diéthylstilbestrol
DMBA	7,12-Dimethylbenz(a)anthracene
E2	Œstradiol
EMT	Transition épithélio-mésenchymateuse/ epithelial-mesenchymal transition
ER	Récepteur aux œstrogènes/ Estrogen receptor
EREs	Estrogen responsive elements
FOXA1	Forkhead box protein A1
FOXOs	Forkhead box class O
FOXM1	Forhead box protein M1
FMO	Monoxygénase contenant des flavines

FUI	Fond Unique Interministériel
GFRs	Facteurs de croissance
GI	Glycéollin I
GII	Glycéollin II
GMCSF	Facteur de stimulation des colonies de macrophages granulocytaires
HER2	Récepteur aux facteurs de croissance épidermiques humains
HGF	Hepatocyte growth factor
HM	Hydrophobic C-terminal regulatory motif
Hsp90	Heat shock protein 90
JNK	Jun N-terminal kinase
LBD	Ligand binding domain
mTOR	Mammalian Target Of Rapamycin
mTORC2	Target of rapamycin complex 2
NACT	Chimiothérapie néoadjuvante
NADPH	Nicotinamide adénine dinucléotide phosphate
NCOR	Nuclear receptor co-repressor
NF1	Neurofibromin 1
OMS	Organisation mondiale de la santé
P-gp	Glycoprotéine P
p23	Prostaglandin E synthase 3
P450	Enzymes du cytochrome P450
p70S6K	Ribosomal protein S6 kinase
PAS	Per-Arnt-Sim/ Period-aryl hydrocarbon receptor nuclear translocator- single minded
PDK1	Phosphoinositide-dependent kinase-1
PHD	Pleckstrin homology domain
PR	Récepteur à la progestérone
RTK	Receptor tyrosine kinase
SERDs	Selective Estrogen Receptor Degradation
SERMs	Selective Estrogen Receptor Modulators
SMRT	Silencing mediator of retinoid and thyroid hormone receptor

TCDD	2,3,7,8-tétrachlorodibenzo-p-dioxine
THS	Traitement hormonal substitutif
TNBC	Triple-negative breast cancer/ Cancer du sein triple négatif
TNF- α	Facteur de nécrose tumorale- α
XAP-2	X-associated protein 2
XRE	Xenobiotic response element

INTRODUCTION

1. Cancer du sein (CS)

1.1. *Glande mammaire et développement du CS*

1.1.1. Glande mammaire

Le sein humain est une unité cutanéoglandulaire composée d'une glande mammaire, de fibres de soutien et de tissu graisseux; le tout est recouvert par la peau. La glande mammaire, dont la principale fonction est la lactation, est divisée en 15 à 20 sections appelées lobes. Chacun est composé de lobules reliés au mamelon par un réseau ramifié de canaux galactophores [1,2] (Figure 1A et 1B). L'épithélium mammaire, qu'il soit canalaire ou lobulaire, est constitué de deux types de cellules : les cellules luminales et les cellules basales (Figure 1C). Les cellules luminales expriment les récepteurs hormonaux tels que les récepteurs aux œstrogènes ou à la progestérone. Elles possèdent une activité de sécrétion du lait dans la lumière du canal galactophore. Les cellules basales possèdent une activité de contraction musculaire permettant le déclenchement mécanique de la sécrétion du lait. L'épithélium est entouré d'une lame basale ainsi que d'un stroma, composé principalement de fibroblastes et d'adipocytes [3] (Figure 1C).

Les deux types cellulaires se différencient par l'expression de marqueurs spécifiques. Les cellules luminales expriment les récepteurs aux œstrogènes (ER), à la progestérone (PR) ou à la prolactine, les cytokératines 8 et 18 ainsi que la molécule de jonction EpCam. Les cellules basales sont caractérisées par l'expression des cytokératines 5, 14 et 17, de la P-cadhérine, des cadhérines desmosomiques et différents marqueurs associés aux muscles lisses [3,4].

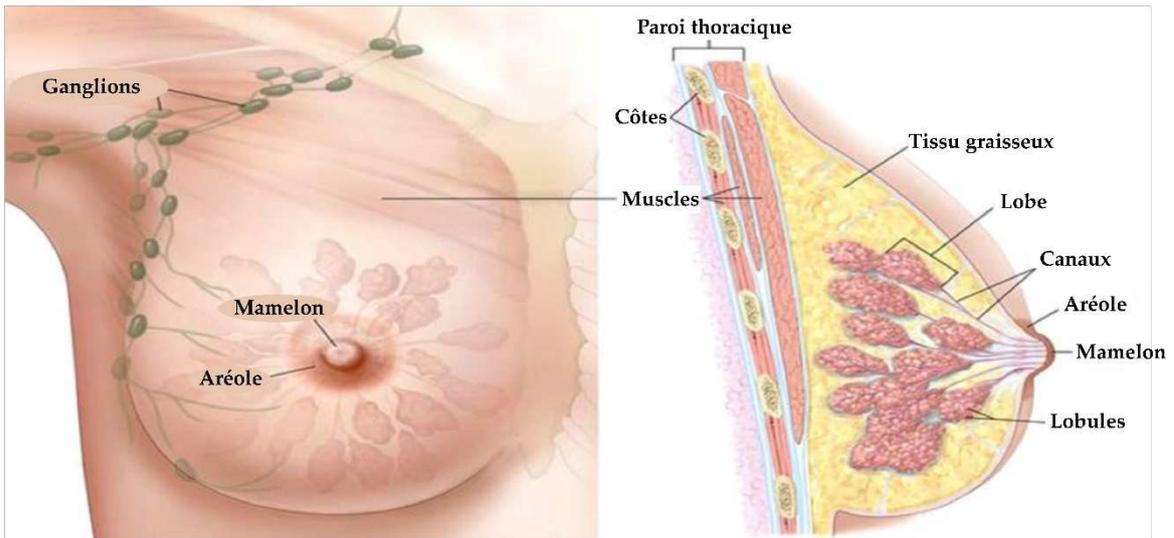
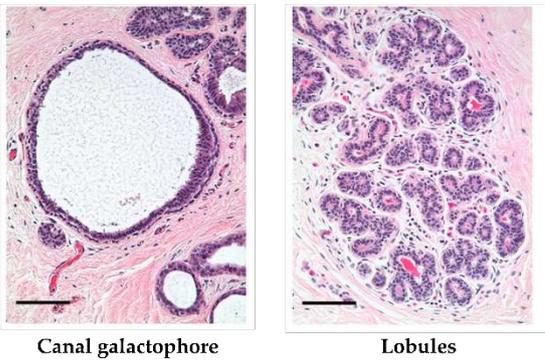
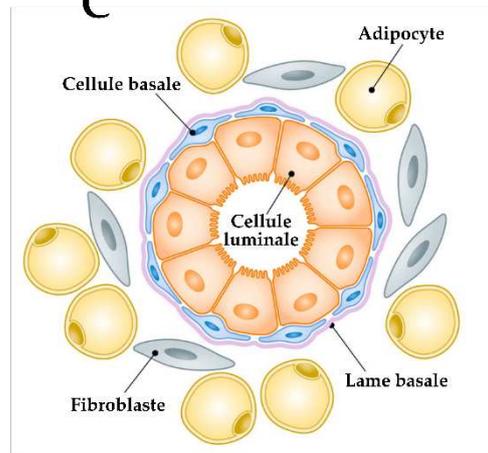
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Figure 1. Glande mammaire humaine. (A) Anatomie du sein chez la femme. Image tirée et traduite de la publication de Singh *et al.* [5]. (B) Coupes histologiques du canal galactophore et des lobules (barre d'échelle = 100 μ m). (C) Epithélium mammaire. Images tirées et traduites de la publication de Pellacani *et al.* [6].

Le développement de la glande mammaire se déroule en majeure partie après la naissance [7]. La première phase de développement post natal est la puberté qui se caractérise par le développement canalaire de la glande. La seconde phase intervient au moment de la gestation, et se caractérise par la différenciation alvéolaire et l'acquisition des propriétés fonctionnelles de la glande [4]. L'ensemble du développement et de la différenciation des cellules mammaires est contrôlé par les principales hormones sexuelles féminines à savoir les œstrogènes, la progestérone et la prolactine. Il est également régulé par d'autres facteurs tels que

les facteurs de croissance. Par exemple, l'amphiregulin (AREG), membre de la famille EGF (Epidermal Growth Factor), est un médiateur essentiel de la fonction des récepteurs des œstrogènes dans le développement de la glande mammaire pendant la puberté [7].

1.1.2. Développement du cancer du sein (CS)

Le développement du CS se déroule classiquement en trois étapes : l'initiation, la promotion et la progression (Figure 2) [4].

L'étape d'initiation correspond à l'apparition, dans une cellule saine, d'altérations génétiques ou épigénétiques conduisant à (i) l'activation de proto-oncogènes favorisant la survie, la prolifération et la migration cellulaire, et (ii) l'inactivation de gènes suppresseurs de tumeurs qui, au contraire, inhibent ces mécanismes cellulaires. Ces altérations de l'expression génétique, qu'ils soient en lien avec des prédispositions génétiques ou qu'ils apparaissent suite à l'exposition à des agents carcinogènes, conduisent à la formation d'une cellule dite initiée. Néanmoins, cette étape d'initiation peut être réversible grâce à l'intervention des mécanismes de réparation de l'ADN [4].

L'étape de promotion correspond à une phase de croissance caractérisée par une prolifération importante à partir de la cellule initiée en réponse aux hormones stéroïdiennes et à différents facteurs de croissance qui favorisent, en plus de la prolifération, l'échappement à l'apoptose. L'étape de promotion aboutit à la formation d'une masse tumorale *in situ* [4].

L'étape de progression correspond à la propagation des cellules de la masse tumorale qui sont transportées par voies sanguines ou lymphatiques. Ces cellules vont coloniser d'autres tissus formant ainsi des tumeurs secondaires, appelées métastases. Cette étape dépend de l'acquisition de propriétés migratrices et invasives qui permettent la transition épithélio-mésenchymateuse (EMT) [8]. Ce processus se caractérise par la perte d'expression de marqueurs épithéliaux au profit de l'acquisition de marqueurs mésenchymateux caractéristiques des cellules plus

invasives. Les sites métastatiques les plus communs issus d'un CS sont le cerveau, l'os, le poumon et le foie [9].

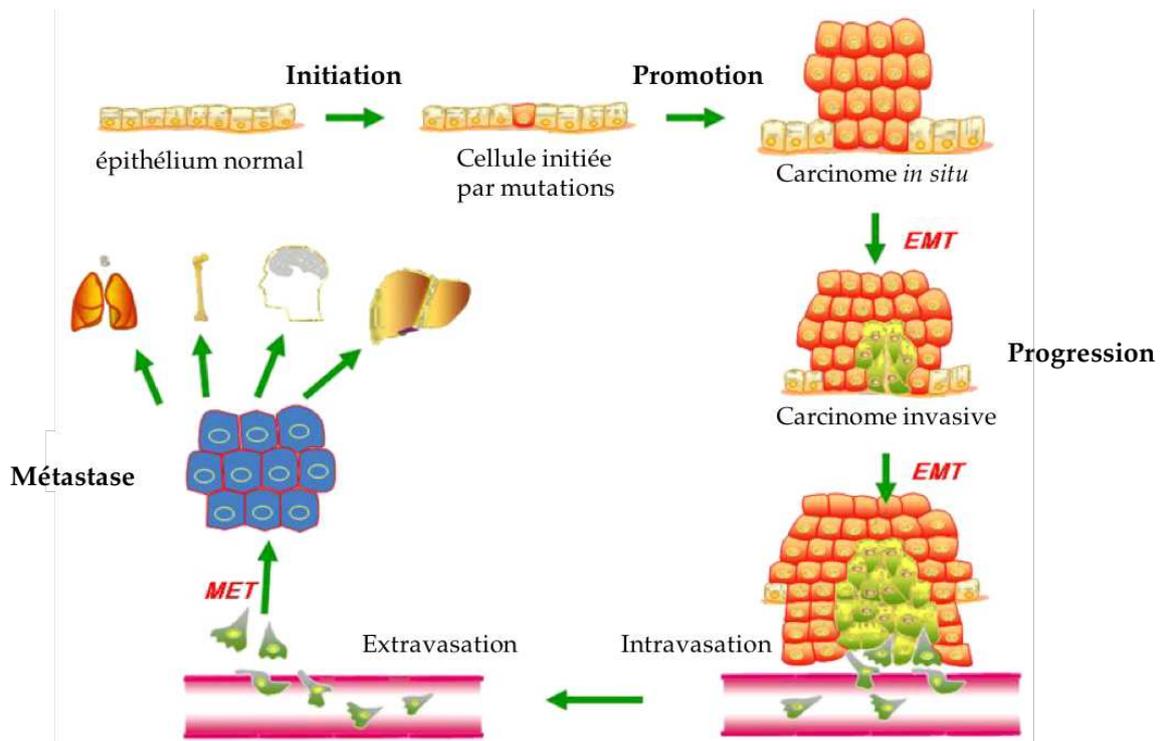


Figure 2. Progression du cancer du sein. EMT: epithelial-mesenchymal transition; MET : mesenchymal-epithelial transition. Figure extraite, traduite et modifiée de l'article de Liu *et al.* [8]

Toutes les cellules tumorales qui composent une même tumeur n'ont pas forcément la même morphologie, la même tumorigénicité, la même sensibilité aux traitements ou encore les mêmes propriétés migratrices et invasives [10]. La prise en compte de cette hétérogénéité permet d'affiner la stratégie thérapeutique à adopter [11].

1.2. Epidémiologie du CS

Le CS est le cancer le plus fréquent chez les femmes. Il est d'ailleurs le cancer le plus souvent diagnostiqué chez les femmes dans 140 pays [12]. En 2018, dans le monde, environ 2,1 millions de femmes ont été diagnostiquées porteuses d'un CS et 626 679 femmes sont décédées de cette maladie [13]. Les taux d'incidence les plus élevés sont signalés dans les pays d'Europe du Nord et de l'Ouest (Danemark, Belgique, Royaume-Uni), l'Amérique du Nord, l'Australie et la Nouvelle-Zélande

(Figure 3). Cependant, le CS ne se limite pas aux pays riches, la mortalité par CS est élevée dans de nombreux pays en voie de développement (Afrique subsaharienne, Pakistan) malgré une incidence plus faible dans ces régions [12] (Figure 3). La raison d'une telle différence du ratio incidence/mortalité est un retard du diagnostic et un accès limité au traitement [9].

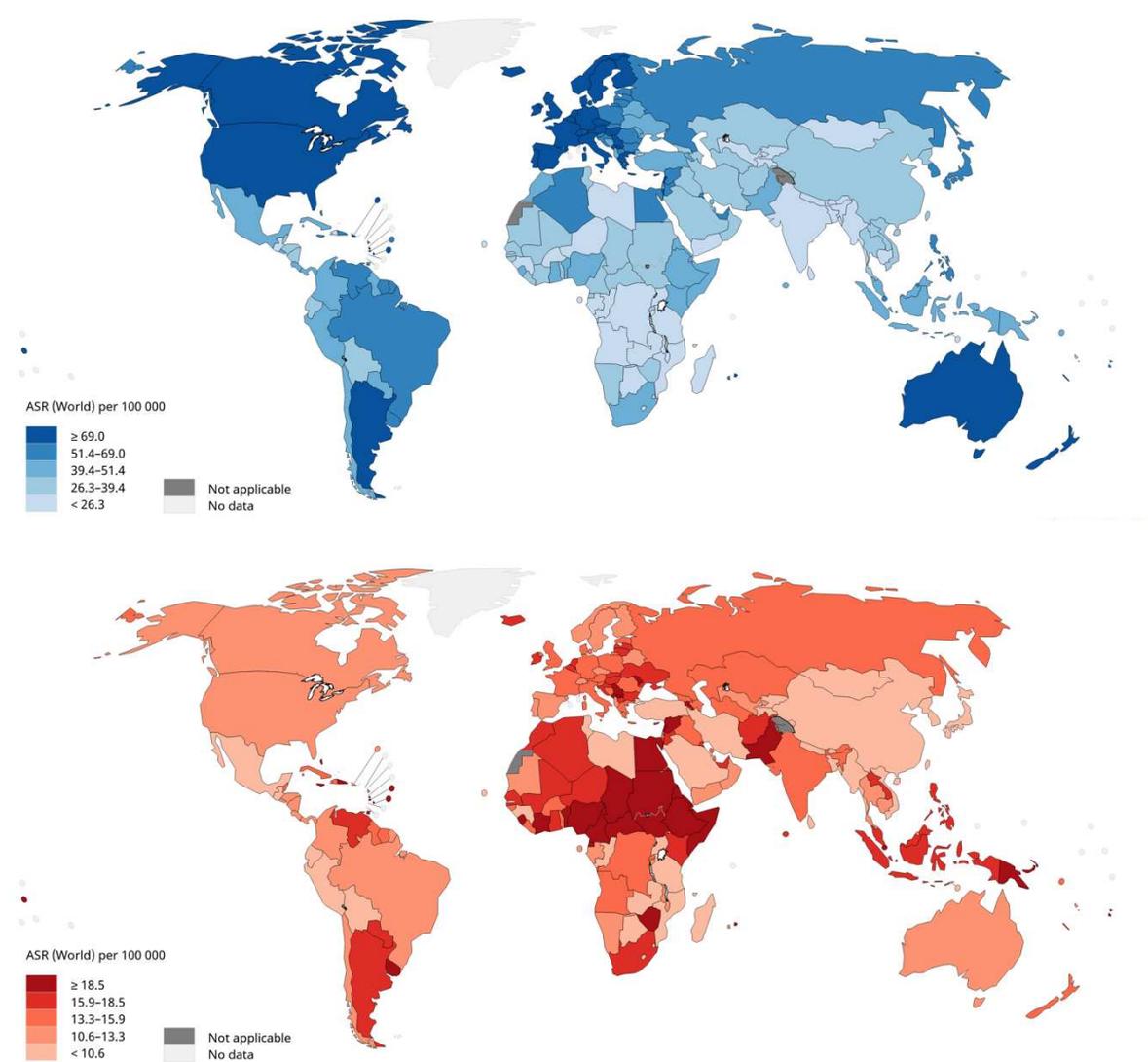


Figure 3. Carte mondiale des taux estimés d'incidence (bleu) et de mortalité (rouge) du cancer du sein chez les femmes en 2018. Les chiffres sont exprimés en taux pour 100 000 personnes. Source : GLOBOCAN (<http://globocan.iarc.fr>)

En France, parmi 177 400 nouveaux cas de cancers chez les femmes en 2018, le CS est le plus fréquent, soit 58 459 cas (INCA). Avec 12 146 décès estimés en 2018, ce cancer est au premier rang des décès par cancer chez la femme. Cependant, la

mortalité de ce cancer en France diminue d'année en année et le taux de survie net à 5 ans est de 87% (INCA).

1.3. Facteurs de risque et de protection

Le CS est une maladie multifactorielle dont certains facteurs reconnus peuvent augmenter ou diminuer le risque de contracter la maladie.

1.3.1. Sexe et âge

Le CS se développe dans 99% des cas chez les femmes mais apparaît aussi chez les hommes dans de rares cas ($\leq 1\%$, INCA).

L'âge est aussi un facteur important. Sur les données observées en France en 2012, l'incidence augmente après 30 ans avec 3 pics : l'un vers 45 ans, un autre vers 70 ans et un troisième vers 85 ans [14].

1.3.2. Génétique

Environ 5 à 10% des CSs sont liés à des mutations génétiques héritées d'un parent. La cause la plus fréquente de CS héréditaire est une mutation du gène BRCA1 ou BRCA2. Statistiquement, les femmes atteintes d'une mutation BRCA1 présentent un risque de développer un CS de 55 à 65% tout au long de la vie. Pour les femmes avec une mutation BRCA2, le risque est de 45%. En moyenne, une femme avec une mutation du gène BRCA1 ou BRCA2 a environ 70% de risque de développer un CS à 80 ans [15].

1.3.3. Facteurs hormonaux endogènes

Nulliparité : Comparées aux femmes nullipares, les femmes qui ont eu au moins une grossesse à terme et non tardive, ont une réduction de risque de CS. La protection a tendance à augmenter avec le nombre d'enfants [16].

Âge au premier enfant : Plus l'âge de la première grossesse est jeune, plus la protection contre le CS est importante. En effet, la grossesse à terme induit la différenciation terminale des glandes mammaires qui les rendent ensuite moins

sensibles à l'effet de divers carcinogènes [17]. Par contre, plus la grossesse est tardive, plus le risque augmente [17]. Il est observé que les femmes qui ont eu leur premier enfant après 30 ans ont un risque supérieur à celui des femmes nullipares.

Allaitement : L'allaitement protège probablement contre le CS mais uniquement dans les cas où cet allaitement est prolongé. Le risque diminue d'environ 4% pour chaque année d'allaitement [14].

Puberté et ménopause : Les femmes auront plus de cycles menstruels si elles commencent leurs menstruations tôt, en particulier avant l'âge de 12 ans et si elles sont ménopausées plus tard, surtout après 55 ans. Elles auront donc une exposition plus longue aux hormones œstrogènes et progestérones, conduisant à un risque légèrement plus élevé de CS [15].

1.3.4. Facteurs hormonaux exogènes

Contraceptifs oraux (CO) : Il est difficile d'établir une relation entre l'utilisation des CO et le CS car la composition et le dosage en œstroprogestatifs a grandement varié au cours du temps et selon les pays. Une augmentation faible de risque de CS a été observée chez les utilisatrices de CO mais ce sur-risque semble disparaître 10 ans après l'arrêt des CO [18]. Un risque relatif de l'ordre de 1,5 est seulement retrouvé pour les femmes ayant utilisé des CO très jeunes pendant au moins 5 ans et avant une première grossesse [19].

Traitement hormonal substitutif (THS) : Les études ont montré une association positive entre l'utilisation d'un THS après ménopause et le risque de CS. Une étude américaine a observé une augmentation significative du risque de CS chez les femmes ménopausées traitées par une association hormonale à base d'œstrogènes conjugués et d'un analogue synthétique de la progestérone [20]. Ce lien a bien été confirmé par une grande étude de cohorte « Million Women Study » chez les femmes britanniques [21]. Suite à ces résultats, la prescription de THS a fortement diminué en France et dans d'autres pays et a entraîné une baisse de l'incidence des cancers du sein post-ménopausiques [14].

1.3.5. Perturbateurs endocriniens et polluants chimiques

Nous sommes exposés à un certain nombre de perturbateurs endocriniens et à des polluants chimiques (comme les pesticides organochlorés) susceptibles de favoriser le développement de tumeurs mammaires. Des études ont montré que l'exposition au bisphénol A, aux parabènes et aux alkylphénols, qui sont présents dans de nombreux produits à usage courant, peut probablement augmenter le risque de CS [22–25]. Nous pouvons aussi noter l'exemple du diéthylstilbestrol (DES), qui était prescrit entre les années 1940 et 1970 aux femmes enceintes pour prévenir les avortements spontanés. Les femmes ayant reçu ce traitement ainsi que leur descendance ont un risque accru de CS [15,26,27].

1.3.6. Alimentation

Actuellement, de plus en plus d'attention est portée sur l'impact de l'alimentation sur la santé, y compris sur le CS. Brièvement, les études montrent qu'une forte consommation de graisses saturées, de cholestérol, de viande rouge, de viande transformée et de plus de cinq œufs par semaine augmente le risque de CS. Inversement, la consommation de certains fruits et légumes, de graisses insaturées de poisson, de yaourt et d'autres produits laitiers peu riches en lipides diminuent le risque de CS [28].

Certains régimes alimentaires se caractérisent par une faible incidence de CS dans la population consommatrice. L'une des raisons qui explique une faible prévalence de CS chez les asiatiques (chinois, japonais) est leur alimentation riche en fruits et légumes, notamment une consommation importante de nourritures à base de soja. Une étude cas-témoins chez les femmes chinoises montre qu'un apport élevé en soja pendant l'adolescence peut réduire le risque de CS plus tard dans la vie [29]. Une autre étude cas-témoins chez les femmes américaines d'origine asiatique a également montré qu'une consommation élevée de soja au cours de l'enfance est associée à une réduction du risque de CS. Ce risque peut être encore réduit par la consommation à l'âge adulte [30]. Une récente étude chinoise réalisée

sur 300.000 femmes confirme l'effet préventif de la consommation élevée du soja contre le CS [31].

Plusieurs études cliniques ont également démontré que le régime méditerranéen pouvait diminuer le risque de CS. Les résultats de l'essai clinique randomisé PREDIMED suggèrent un effet bénéfique d'un régime méditerranéen supplémenté avec de l'huile d'olive extra-vierge dans la prévention primaire du CS bien que des études à plus long terme et sur une plus grande population soient nécessaires pour confirmer cet effet [32]. Une méta-analyse ultérieure de 83 études d'observation et d'essais randomisés incluant 2 130 753 sujets et différents types de cancers confirme que le régime méditerranéen est inversement associé au CS [33]. Une étude cas-témoins en Italie et en Suisse a trouvé la même association [34]. Une méta-analyse concentrée sur le CS ER-négatif post-ménopausique a également suggéré une relation inverse entre le régime méditerranéen et le CS [35]. Bien que les mécanismes de l'effet protecteur du régime méditerranéen dans le CS ne soient pas encore compris, certaines études suggèrent que cette association pourrait s'expliquer par l'effet modulateur de ce régime sur l'inflammation chronique [36].

1.3.7. Poids et taille

Le surpoids ou l'obésité à l'âge adulte augmentent le risque de CS post-ménopausique mais cette association n'est pas retrouvée pour les cancers survenant avant la ménopause [14]. Cette observation peut être expliquée par le fait qu'après la ménopause, les ovaires cessent de produire des œstrogènes, la plupart des œstrogènes d'une femme ménopausée proviennent alors du tissu adipeux. Ainsi, la présence importante de tissu adipeux après la ménopause augmentera les niveaux d'œstrogènes et augmentera ainsi le risque de CS. De plus, le surpoids a tendance à entraîner des taux sanguin d'insuline plus élevés, et des taux d'insuline plus élevés sont liés à certains cancers, dont le CS. Néanmoins, le lien entre le poids corporel et le risque de CS est complexe et reste à étudier [15].

Concernant la taille, une étude à long terme basée sur 22 931 Norvégiennes a mis en évidence une association positive de la taille à la naissance avec le risque de CS, qui peut être particulièrement forte chez les femmes dont la mère était grande [37]. Chez les adultes, la taille est un facteur de risque de CS post-ménopausique, mais chez les femmes préménopausées, cette relation est moins claire [38]. Il est possible que les facteurs liés à la taille, tels que les facteurs hormonaux et génétiques, stimulent le développement et la progression du cancer [39].

1.3.8. Alcool et tabagisme

Alcool : La consommation d'alcool est clairement liée à un risque accru de CS. L'augmentation du risque causée par ce facteur est en corrélation avec la quantité consommée [15,40,41]. Le risque est statistiquement significatif à des niveaux de consommation aussi faibles que 5,0 à 9,9 g d'alcool par jour, équivalant à 3 à 6 verres par semaine [40].

Tabagisme actif et passif : Le tabagisme, qu'il soit actif ou passif, augmente le risque de CS [42]. L'arrêt du tabagisme chez les femmes atteintes de CS réduit leur risque de mortalité [43].

1.3.9. Activité physique

L'activité physique régulière, en particulier chez les femmes après la ménopause, peut réduire le risque de CS et semble associée à une diminution de 30% du risque de mortalité [44,45]. On ne sait pas exactement comment l'activité physique pourrait réduire le risque de CS, mais cela peut être dû au fait que l'activité physique a un effet sur le poids corporel, l'inflammation, les hormones et l'équilibre énergétique [15].

1.4. Classification du CS

Les caractéristiques histologiques et moléculaires ont des implications importantes pour la thérapie. Plusieurs classifications du CS sur la base des caractéristiques moléculaires et histologiques ont été développées. La figure 4

présente la classification intrinsèque de substitution, développée à partir de celle décrite par Perou *et al.* en 2000 [46], généralement utilisée en clinique. Elle est basée sur l'histologie et l'expression immunohistochimique des protéines clés: récepteur aux œstrogènes (ER), récepteur à la progestérone (PR), récepteur aux facteurs de croissance épidermiques humains (HER2) et marqueur de prolifération (Ki67). Cette classification divise le CS en 5 sous-types : Triple negative, HER2-enriched (non-luminal), luminal B-like HER2+, luminal B-like HER2- et luminal A-like (Figure 4).

Sous-type « luminal A-like »: Les tumeurs appartenant à ce sous-type expriment fortement les récepteurs ER et PR, mais n'expriment pas le récepteur HER2. Ce sous-type, qui représente 60 à 70% des cancers du sein, est associé à un faible taux de prolifération et a un bon pronostic.

Sous-type « luminal B-like HER2- »: Les tumeurs de sous-type « luminal B-like HER2- » expriment les récepteurs ER et PR mais moins fortement que le sous-type « luminal A-like » et n'expriment pas le récepteur HER2. Ce sous-type, qui représente 10 à 20% des cancers du sein, est associé à un taux de prolifération plus élevé que le sous-type « luminal A-like » et a un pronostic intermédiaire.

Sous-types « luminal B-like HER+ » et « HER2-enriched (non-lumial) »: Les tumeurs de ces deux sous-types, qui représentent 13 à 15% des cancers du sein, sont caractérisées par l'amplification du gène codant pour le récepteur HER2. Le sous-type « luminal B-like HER+ » exprime faiblement les récepteurs ER et PR alors que le sous-type « HER2-enriched (non-lumial) » n'exprime pas ces deux récepteurs hormonaux. Ces deux sous-types ont un taux de prolifération plus important que les sous-types luminaux et sont associés à un pronostic intermédiaire. Le sous-type « HER2-enriched (non-lumial) » est agressif mais répond aux thérapies ciblées.

Sous-type « triple négative »: Les tumeurs appartenant à ce sous-type n'expriment ni ER ni PR ni HER2. Ce sous-type, qui représente 10 à 15% des cancers du sein, est très agressif et associé à un mauvais pronostic.

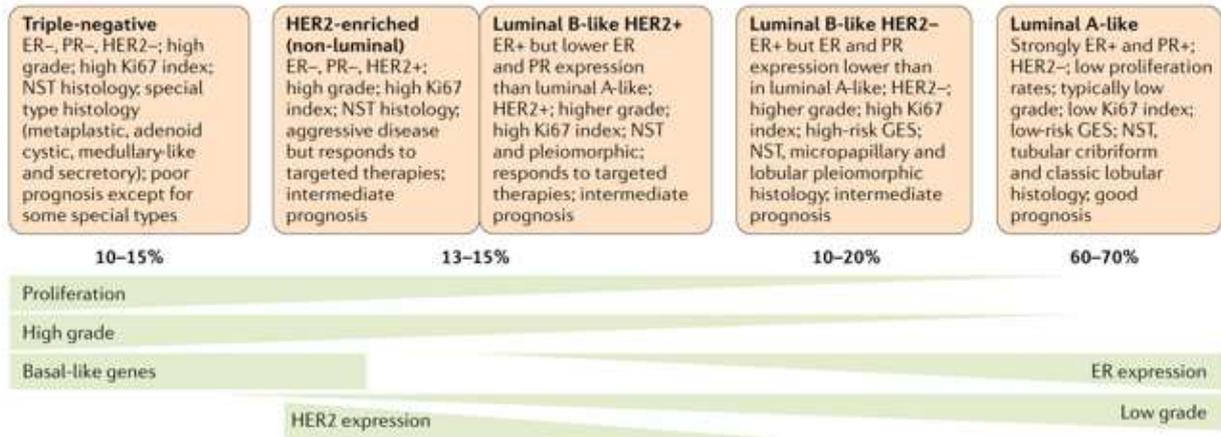


Figure 4. Classification du cancer du sein basée sur l'histologie et l'expression des protéines clés. ER : estrogen receptor, PR : progesterone receptor, HER2 : human epidermal growth factor receptor 2, NST :no specific type, Ki67 : prolifération marker, GES : gene expression signature, - : négative, + : positive. Figure extraite et modifiée de l'article de Harbeck *et al.* [9].

1.5. Les récepteurs hormonaux

1.5.1. Récepteurs aux œstrogènes (ER)

Les récepteurs aux œstrogènes ER α et ER β (Figure 5A) font partie de la superfamille des récepteurs nucléaires dépendants du ligand, partageant l'organisation structurelle et fonctionnelle commune des autres membres de cette famille [47]. Ils possèdent tous 3 régions subdivisée en 6 domaines, de A à F (Figure 5A).

Le domaine A/B, situé à l'extrémité N-terminale d'ER, contient le domaine de transactivation AF-1 (activation function 1) qui permet d'activer la transcription de façon ligand-indépendante [48]. De plus, ce domaine possède plusieurs sites de phosphorylation et de sumoylation qui permettent le recrutement de co-activateurs ou de co-répresseurs pouvant moduler la liaison à leurs éléments de réponse spécifiques [47].

La région centrale du récepteur abrite le domaine C, également appelé DBD (DNA Binding Domain) et le domaine D, une région charnière. Le DBD assure la liaison d'ER aux éléments de réponse spécifiques localisés dans la région promotrice des gènes cibles dits EREs (estrogen responsive elements), tandis que la région

charnière D est importante pour médier la signalisation de certaines kinases intracellulaires qui régulent l'activité et les fonctions d'ER [49].

L'extrémité C-terminale d'ER, le domaine E/F, contient la fonction d'activation 2 (AF-2 - activation function 2) et le domaine de liaison au ligand (LBD- ligand binding domain). Ce domaine est responsable de l'activation dépendante du ligand dans la régulation de l'expression des gènes.

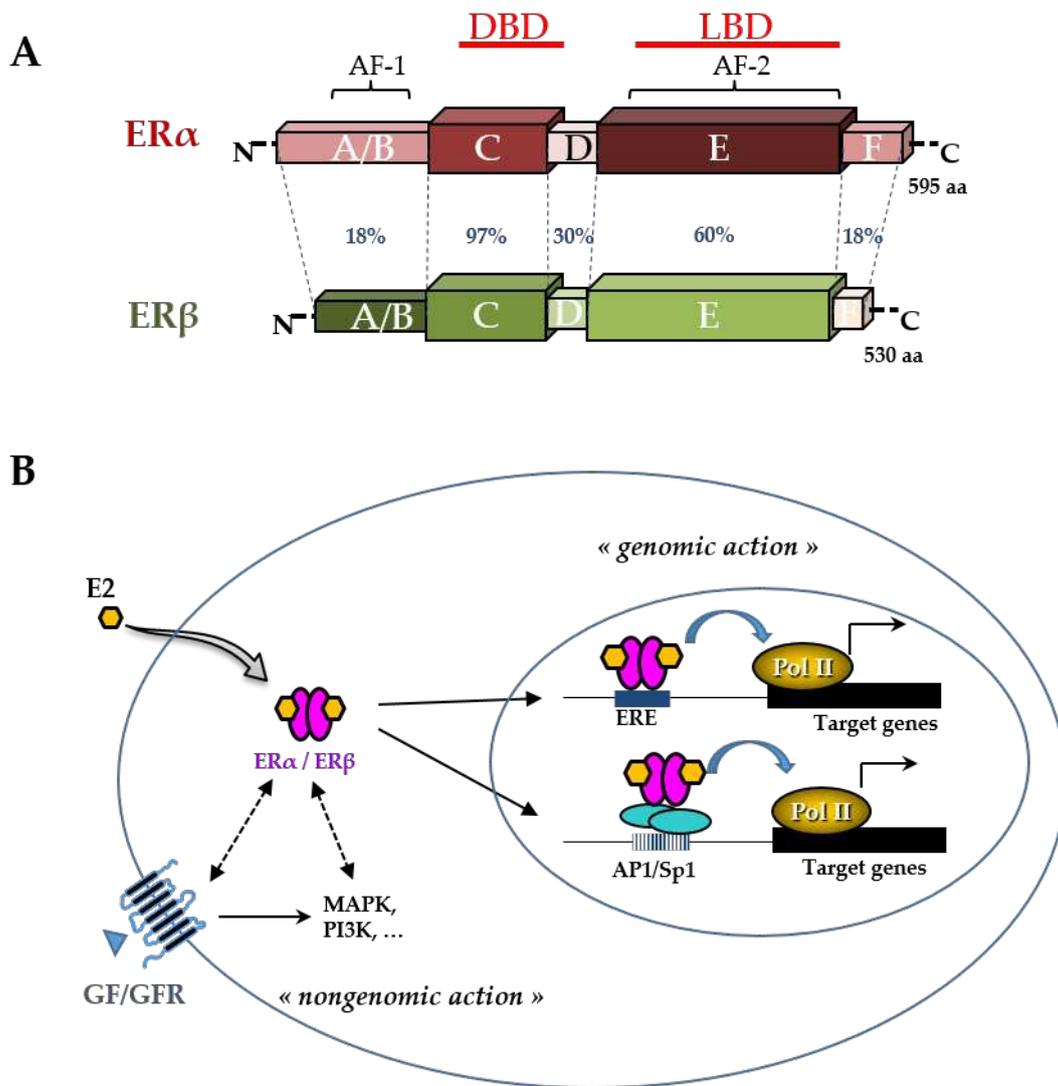


Figure 5. Structure et mécanisme d'action du récepteur des œstrogènes. (A) Les structures schématiques des deux ER α et ER β humains et le pourcentage d'homologie entre les différents domaines (annotés par les lettres A à F) sont indiqués. Les domaines DBD (DNA binding domain), LBD (Ligand Binding Domain), AF-1 (activation function 1) et AF-2 (activation function 2) sont présentés. Le nombre d'acides aminés pour chaque récepteur est également indiqué sur le côté droit. (B) L'œstradiol (E2) intervient dans de nombreux effets phénotypiques dans les cellules en se liant aux ERs et en les activant. E2 pénètre librement dans la cellule par les membranes lipidiques et se lie à ER, présent dans le cytoplasme et/ou dans le noyau. ER activé forme un dimère qui se fixe à ses éléments de

réponse au niveau chromatine EREs (estrogen responsive elements) ou aux sites de la protéine AP1 (activator protein 1) ou de la protéine Sp1 (specificity protein 1). ER est ensuite capable de remodeler la chromatine en recrutant des cofacteurs et en activant l'ARN polymérase II (Pol II), au niveau des gènes cibles (action génomique). En outre, les ER peuvent générer un effet non génomique rapide grâce à l'interaction avec les kinases intracellulaires telles que MAPK (mitogen-activated protein kinase) et PI3K (phosphatidylinositol 3-kinase) et les voies du récepteur du facteur de croissance (GFR). Figure tirée et traduite de notre publication [50].

ER est activé de façon ligand-dépendante par les œstrogènes dont l'E2 qui exerce l'activité œstrogénique la plus forte. L'activité d'ER comprend les fonctions génomiques et non génomiques (Figure 5B). Les fonctions nucléaires génomiques comprennent une activité classique, où ER se lie aux EREs, et une activité non classique, où ER est lié à d'autres facteurs de transcription, fonctionnant comme un co-régulateur pour moduler l'activité transcriptionnelle via différents sites, tels qu'AP1 et Sp1 [51]. Les fonctions non génomiques d'ER se produisent à l'extérieur du noyau, où ER interagit directement ou indirectement avec les récepteurs des facteurs de croissance (GFRs) et avec d'autres molécules de signalisation, entraînant l'activation des voies de signalisation en aval telles que les voies PI3K et MAPK. Cette signalisation régule la transcription des gènes conduisant à la prolifération, à la survie et à d'autres caractéristiques tumorales clés telles que l'angiogenèse et la migration.

1.5.2. Récepteurs à la progestérone (PRs)

Les PRs appartiennent également à la superfamille des récepteurs nucléaires [47]. Les PRs sont activés par la progestérone qui est principalement produite par le corps jaune dans les ovaires pendant la seconde moitié du cycle menstruel. Ces récepteurs sont responsables du développement de certains tissus spécialisés dans le sein et dans l'appareil reproducteur [52].

Le rôle de la progestérone dans le CS n'est pas clair, mais il a été suggéré que la progestérone est une source importante d'auto-renouvellement cellulaire dans le tissu mammaire, et qu'une activité de PR non contrôlée dans le tissu précancéreux contribuerait au développement de tumeur [52]. Dans le CS, ER régule l'expression

de PR, donc la présence de PR indique que la voie de signalisation d'ER fonctionne. L'expression de PR est donc utilisée comme indicateur d'une bonne réponse aux hormonothérapies [53]. Les expressions d'ER et de PR sont parmi les indicateurs les plus utiles pour prédire une réponse (ou un manque de réponse) aux thérapies adjuvantes existantes.

1.6. Options actuelles de traitements

La prise en charge du CS est multidisciplinaire, elle consiste par des approches à visée locale (chirurgie et radiothérapie) ou systémique. Les thérapies systémiques incluent la thérapie endocrinienne pour la maladie à récepteurs hormonaux positifs, la chimiothérapie, la thérapie anti-HER2 pour la maladie à HER2 positive, d'autres types de thérapies ciblées et l'immunothérapie. Récemment, les concepts de traitement dans le CS ont évolué pour prendre en compte l'hétérogénéité de la tumeur au niveau moléculaire, l'accent étant mis sur des thérapies plus dirigées biologiquement et une désescalade thérapeutique afin de réduire les effets indésirables du traitement. De plus, certaines caractéristiques, telles que la taille et la localisation de la tumeur et des métastases, peuvent également influencer le choix de la thérapie [9].

1.6.1. Traitements à visée locale

1.6.1.a. Chirurgie

La chirurgie est le traitement utilisé en première intention pour la prise en charge de la plupart des cancers du sein. Une chirurgie conservatrice (tumorectomie) qui consiste à retirer uniquement la tumeur ainsi qu'une marge d'exérèse correspondant à une petite partie de tissu sain avoisinant, est réalisée lorsque la tumeur est bien localisée et de petite taille. En revanche, une chirurgie non conservatrice (mastectomie), qui correspond à l'ablation complète du sein, doit être pratiquée lorsque la masse tumorale est trop importante ou s'il s'agit d'une tumeur infiltrante. Bien que la chirurgie conservatrice soit préférable, la mastectomie reste

un choix fréquent pour de nombreuses femmes [54]. Pour privilégier la chirurgie conservatrice, un traitement néo-adjuvant peut être effectué afin d'essayer de réduire la taille de la tumeur. Étant donné que la reconstruction mammaire après la chirurgie a des bénéfices prouvés sur la qualité de vie des patientes [55,56], il est essentiel que les femmes aient la possibilité d'avoir une discussion avec l'équipe médicale sur la reconstruction mammaire avant une mastectomie [57].

Quel que soit le type de chirurgie pratiqué, il est souvent suivi de la mise en place de traitements adjuvants (radiothérapie, chimiothérapie et/ou hormonothérapie) qui ont pour objectif de diminuer le risque de récurrence.

1.6.1.b. Radiothérapie

La radiothérapie externe consiste à diriger précisément sur la zone à traiter des rayons ionisants afin d'endommager les cellules cancéreuses mammaires et de bloquer leur prolifération. La radiothérapie est principalement utilisée en seconde intention après une chirurgie pour améliorer la survie globale et prévenir le risque de rechute des patientes par l'élimination des cellules tumorales résiduelles. Cette thérapie peut également être utilisée pour traiter les métastases osseuses et cérébrales [58,59].

1.6.2. Traitements à visée systémique

1.6.2.a. Hormonothérapie

L'hormonothérapie du CS peut être adjuvante (après la chirurgie) ou néoadjuvante (avant la chirurgie). La thérapie endocrinienne adjuvante joue un rôle clé dans le traitement du cancer ER-positif. Elle peut être utilisée sur une durée de 5 à 10 ans.

L'objectif de ce traitement est de bloquer l'effet des hormones féminines endogènes qui stimulent la croissance de la tumeur mammaire. Pour cela plusieurs stratégies ont été développées.

La première stratégie, qui est non médicamenteuse, consiste à réaliser une ablation ou une irradiation des ovaires afin d'empêcher la production des hormones féminines endogènes chez les patientes non ménopausées. Bien que très efficaces, ces méthodes induisent une ménopause précoce chez des femmes jeunes en âge de procréer.

Parallèlement, plusieurs stratégies d'hormonothérapie médicamenteuse ont été développées. Ces stratégies sont présentées dans la figure 6.

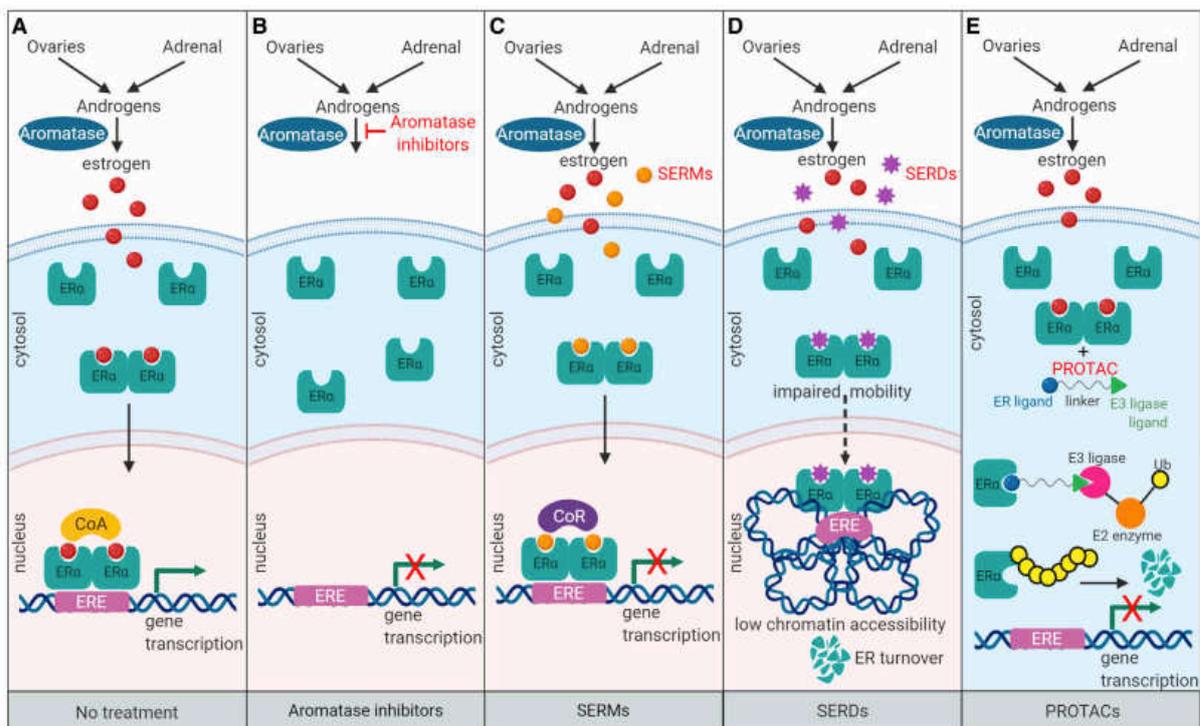


Figure 6. Mécanisme d'action des thérapies endocriniennes

(A) Les ovaires, les glandes surrénales, le tissu adipeux, le sein et d'autres tissus produisent des androgènes, qui sont convertis en œstrogènes par l'aromatase. Lors de la liaison à l'œstrogène, le récepteur aux œstrogènes (ER) se dimérise et se déplace vers le noyau, où les dimères d'ER se lient aux coactivateurs (CoA) pour former un complexe d'ER transcriptionnellement actif.

(B) Les inhibiteurs de l'aromatase (IA) non stéroïdiens et réversibles tels que le létrozole ou l'anastrozole, ou les IA stéroïdiens irréversibles tels que l'exémestane, bloquent la production d'œstrogènes en inhibant l'aromatase des androgènes en œstrogènes.

(C) Les SERMs (Selective estrogen receptor modulators) tels que le tamoxifène et le raloxifène inhibent de manière compétitive la liaison des œstrogènes aux ERs. Les dimères d'ER liés au SERM interagissent avec la chromatine au niveau des éléments de réponse aux œstrogènes (EREs). Cependant, les dimères d'ER liés au SERM s'associent avec des corépresseurs (CoR), qui inhibent l'activité transcriptionnelle des ERs dans le sein.

(D) Les SERDs (Selective estrogen receptor downregulators) tels que le fulvestrant sont considérés comme de purs antagonistes des ERs. L'effet inhibiteur des SERD a récemment

été attribué à la capacité réduite des ERs liés au SERD de se translater vers le noyau. De plus, le complexe ER-SERD est incapable d'établir une conformation de chromatine ouverte pour faciliter la transcription des gènes régulés par ER. ER lié au SERD subit une dégradation en raison d'une mobilité réduite.

(E) Les PROTACs (Proteolysis targeting chimeras) sont des molécules hétérobifonctionnelles qui se composent d'un ligand pour ER et d'un autre ligand, qui sert de substrat au complexe d'ubiquitine ligase E3. Lors de la liaison à l'ER, les PROTAC recrutent le complexe d'ubiquitine ligase E3, qui polyubiquitine ER et le marque pour la dégradation protéasomique.

Figure tirée de publication de Hanker et al. [60]

1.6.2.b. Chimiothérapie

La chimiothérapie désigne les traitements médicamenteux ayant pour but la destruction des cellules cancéreuses par des mécanismes non spécifiques. L'utilisation de chimiothérapie comme adjuvant après la chirurgie existe depuis plusieurs décennies et son utilisation suit un protocole créé par les experts basé sur des propriétés de la tumeur et d'autres facteurs. Dans le passé, la chimiothérapie néoadjuvante (NACT) était réservée aux cas de CS localement avancés, inopérables ou inflammatoires. Plus récemment, des avantages du NACT ont été observés dans le CS opérable précoce [57].

Généralement, il y a 4 grandes familles d'agents de chimiothérapie à savoir les agents alkylants, les taxanes, les anthracyclines et les anti-métabolites, qui présentent différents modes d'action mais qui aboutissent tous au blocage de la division cellulaire. Du fait que leurs effets sont peu spécifiques, de nombreux effets secondaires sont observés: chute des cheveux, nausées et vomissements, modifications de la peau et des ongles... Cela peut même aller jusqu'à l'apparition d'une toxicité hématologique et neurologique. Il est aussi notable qu'un des effets secondaires importants de la chimiothérapie est l'insuffisance ovarienne prématurée chez les femmes préménopausées. Pour les femmes souhaitant préserver leur fertilité, il est recommandé de consulter avant la chimiothérapie un spécialiste de la fertilité pour discuter de la possibilité de cryoconservation d'ovocytes ou d'embryons [61,62].

1.6.2.c. Thérapies ciblant HER2

Dans le sous-type HER2-positif, HER2 accélère la croissance et la division des cellules cancéreuses de façon agressive. Ainsi le blocage des récepteurs HER2 dans les tumeurs HER2-positives inhibe la croissance tumorale. Chez les patientes atteintes d'un CS HER2-enrichi, l'utilisation du trastuzumab, un anticorps monoclonal qui empêche la signalisation intracellulaire d'HER2, améliore la survie sans maladie et la survie globale [63]. Cependant, une étude a montré que le traitement par trastuzumab est associé à un risque 2 fois plus élevé d'insuffisance cardiaque tardive par rapport au traitement de chimiothérapie seul [64]. L'ajout de pertuzumab (un autre anticorps monoclonal dirigé contre le récepteur HER2) au traitement avec le trastuzumab a permis une meilleure survie sans maladie dans les essais ; cependant, il provoque des épisodes de diarrhée importants [65].

1.6.2.d. Autres types de thérapies ciblées

D'autres thérapies ciblant des acteurs impliqués dans la progression des tumeurs ont également été développées. On peut citer des inhibiteurs des enzymes CDK (Cyclin Dependant Kinase) 4 et 6 impliquées dans la régulation du cycle cellulaire, des inhibiteurs enzymatiques d'une poly (ADP-ribose) polymérase et des inhibiteurs de la voie PI3K/Akt/mTOR. Une autre stratégie consiste à cibler le processus d'angiogenèse qui est impliqué dans non seulement la croissance mais aussi dans des métastases de tumeurs [4].

1.6.2.e. L'immunothérapie

L'immunothérapie, qui est principalement basée sur le point de contrôle immunitaire cible, a révolutionné le traitement des tumeurs malignes. Cette thérapie émerge comme une nouvelle approche contre le CS à l'étape de métastase [66]. La monothérapie des immuno-agents contre PD1/PD-L1 ou CTLA-4 a montré son efficacité chez un petit nombre de patientes atteintes de CS triple négatif et métastatique [66,67]. Pour optimiser l'utilisation de l'immunothérapie, de nombreux

essais cliniques ont été mis en place afin d'étudier la combinaison de l'immunothérapie avec la chimiothérapie, la radiothérapie, les thérapies ciblées [66].

1.7. Mécanismes de résistance endocrine

Bien que la plupart des CSs ER-positifs puissent initialement répondre aux traitements endocriniens, 15 à 20% des tumeurs sont intrinsèquement résistantes aux traitements et 30 à 40% acquièrent une résistance aux traitements sur une période de plusieurs années [68]. Chez les patientes atteintes d'un CS traitées au tamoxifène, environ 40% acquièrent une résistance endocrinienne [69]. La résistance aux traitements entraîne inévitablement des rechutes et des métastases, conduisant à la mort. Par conséquent, il est essentiel d'acquérir une compréhension plus complète des mécanismes de résistance et d'élucider les cibles d'une intervention thérapeutique.

Bien que la résistance endocrinienne entraîne une activité ligand-indépendante d'ER, plus de 80% des cas continuent à exprimer ER α , ce qui corrobore plutôt l'hypothèse d'une modulation de son activité que celle d'une extinction [70]. Plusieurs mécanismes ont été suggérés pour expliquer la résistance endocrinienne. Ils sont résumés dans la figure 7 et seront discutés ci-dessous.

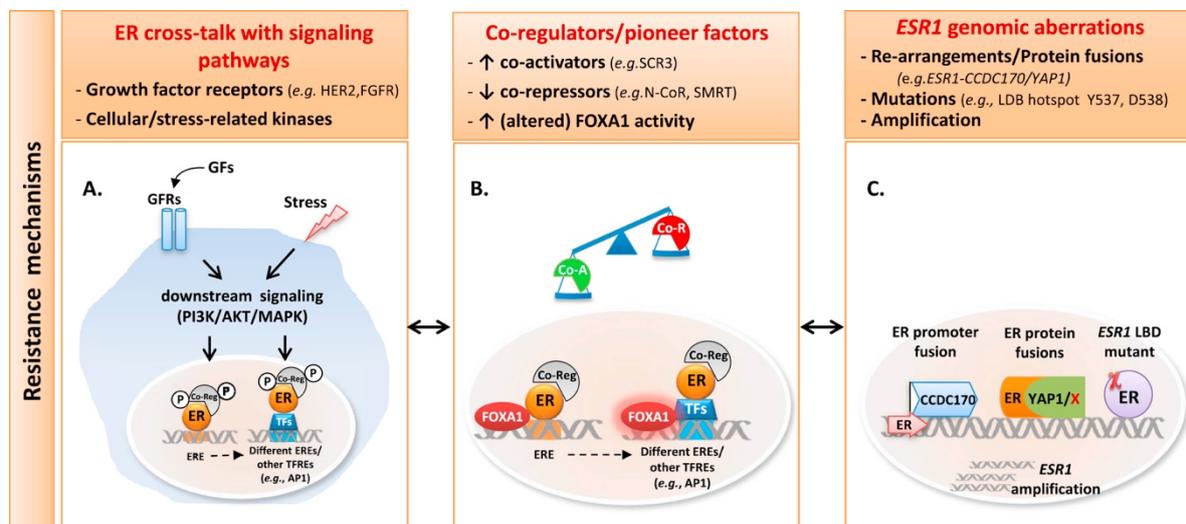


Figure 7. Mécanismes de résistance endocrinienne et de modification des fonctions d'ER dans le cancer du sein. Figure extraite de l'article de Nardone *et al.* [49]

1.7.1. Mécanismes de résistance *via* des voies de signalisation “cross-talk” avec ER

L'interaction de la signalisation œstrogénique avec la voie des facteurs de croissance et des voies kinases joue un rôle important dans le développement de résistance à l'hormonothérapie (Figure 7A).

L'amplification HER2 (ERBB2) est connue depuis 2000 pour réduire la sensibilité aux traitements anti-œstrogéniques [71]. Les études récentes ont montré que des mutations activant HER2 sont impliquées dans la résistance intrinsèque et acquise aux thérapies endocriniennes [72,73] et se retrouvent dans environ 5,8 % des cas de CS non HER2-amplifiés résistants aux hormonothérapies [74]. L'amplification d'autres facteurs de croissance comme EGFR et FGFR est aussi rapportée parmi des cas de CS résistants aux hormonothérapies [74,75]. En outre, les altérations fonctionnelles de NF1 (neurofibromin 1) entraînent une activité RAS constitutive et sont associées à la résistance intrinsèque et acquise à la thérapie endocrinienne [76]. L'altération de ces facteurs est associée à des modifications de voies de signalisations comme PI3K/Akt et MAPK/ERK entraînant une activité ligand-indépendante d'ER α (Figure 8).

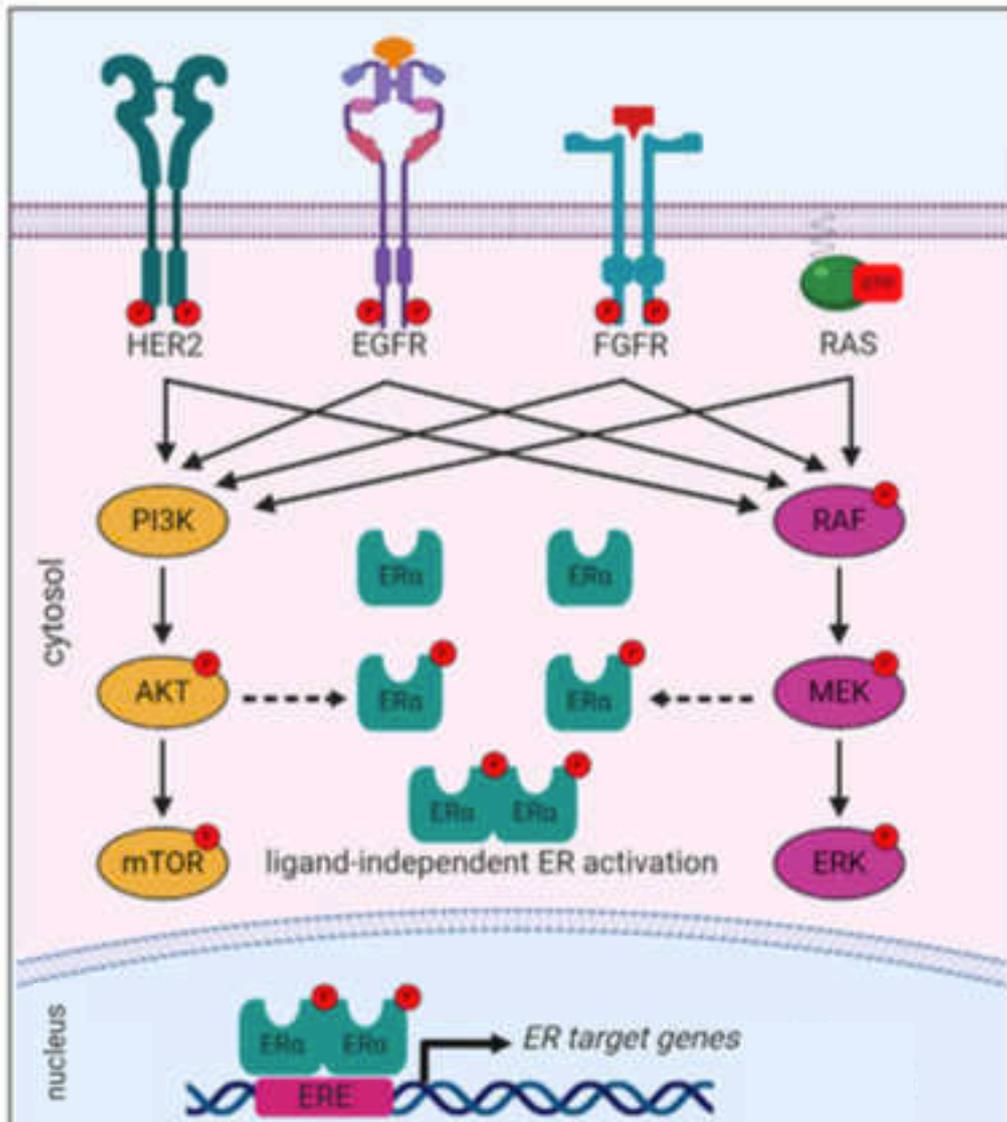


Figure 8. Activation de HER2, EGFR, FGFR et d'autres récepteurs tyrosine kinases favorise la résistance endocrinienne. L'activation aberrante des récepteurs tyrosine kinases (le plus souvent par mutation ou amplification) et l'activation de RAS augmente la signalisation PI3K et MAPK, qui induit la phosphorylation d'ER. La phosphorylation d'ER favorise la transcription des gènes régulés par ER d'une manière indépendante du ligand. Figure extraite et modifiée de l'article de Hanker *et al.* [60]

Dans notre étude, nous nous intéressons aux voies PI3K/Akt. Cette voie et son rôle dans la résistance endocrinienne seront discutés dans la section 1.8.

1.7.2. Mécanismes de résistance *via* des co-régulateurs / facteurs pionniers

Lors de la liaison à ses ligands, ER change de conformation, exposant les surfaces d'interaction pour le recrutement de facteurs accessoires également connus comme co-régulateurs de la machinerie de transcription [77]. Selon la nature du ligand, ER

recrute et interagit avec des protéines qui peuvent soit augmenter (co-activateurs) soit réprimer (co-répresseurs) son activité transcriptionnelle (Figure 7B). La surexpression de SRC3 (aussi connu sous le nom AIB1, amplified-in-breast-cancer-1) est associée à une résistance préclinique et clinique au tamoxifène et entraîne un mauvais pronostic vital [78,79]. De même, une diminution des niveaux des co-répresseurs comme NCOR (nuclear receptor co-repressor) et SMRT (silencing mediator of retinoid and thyroid hormone receptor) est corrélée avec l'acquisition d'une résistance au tamoxifène [80,81].

En plus des co-régulateurs, l'activité transcriptionnelle des ERs est également régulée par des facteurs pionniers. Les facteurs pionniers sont des facteurs de transcription qui peuvent se lier directement à la chromatine et permettent d'ouvrir la chromatine condensée pour aider à la liaison d'autres facteurs de transcription, tels qu'ER et d'autres récepteurs nucléaires. FOXA1 (forkhead box protein A1) est un facteur pionnier impliqué dans le remodelage de la chromatine et il a été démontré qu'il coopère avec ER pour induire l'expression des gènes [82]. Il est intéressant de noter que les mutations du hotspot du promoteur du gène FOXA1 qui augmentent l'expression de FOXA1 sont associées à une sensibilité réduite au fulvestrant [83]. L'amplification ou la surexpression du gène FOXA1 implique l'activation d'un ensemble unique de gènes qui peuvent médier la résistance endocrinienne [84].

1.7.3. Mécanismes de résistance *via* des aberrations génomiques d'ESR1

ER α continue à s'exprimer dans la majorité des cas de résistance endocrinienne acquise. Cependant, la fonction d'ER α est modifiée suite à des thérapies endocriniennes à long terme [85]. Récemment, le séquençage du génome entier et le séquençage ciblé de nouvelle génération ont révélé de multiples aberrations génomiques du locus ER α , y compris des ré-arrangements, des mutations ponctuelles et une amplification génique (Figure 7C).

Il a récemment été démontré que le gène ESR1 codant ER α peut être réarrangé avec ses gènes adjacents. La protéine de fusion ER-CCDC170, exprimée préférentiellement dans le sous-type du CS luminal B, renforce la motilité, la formation de tumeurs et la résistance endocrinienne [86]. D'autres partenaires récemment décrits pour la fusion avec ESR1 sont YAP1 et PCDH11X. Ces deux fusions entraînent une prolifération indépendante des œstrogènes et activent un programme de transcription associé aux métastases [87].

Des études génétiques récentes ont montré que la fréquence élevée des mutations du gène ESR1 (environ 20%) se produit dans les tumeurs résistantes acquises après l'hormonothérapie [88]. Plusieurs mutations ponctuelles d'ESR1 sont identifiées dans le domaine LBD, au niveau de quelques acides aminés à l'intérieur ou à proximité de la région de l'hélice 12 du LBD [89–91]. Les acides aminés Tyr537 et Asp538 sont les « hotspots » des mutations d'ESR1 [70,92,93]. Ces mutations d'allèle unique n'affectent pas la dimérisation d'ER α , mais ils augmentent constitutivement l'activité transcriptionnelle d'ER α [70,94]. En effet, la plupart des mutations d'ER α rapportées confèrent une activité constitutive indépendante du ligand, ce qui entraîne une perte de réponse au tamoxifène et au fulvestrant.

Une autre altération génomique potentiellement importante concerne l'amplification du gène ESR1. Cependant, plusieurs études contradictoires coexistent dans la littérature. Certaines études montrent une amplification non négligeable du gène ESR1 dans les cellules cancéreuses, par exemple 5,9% d'après Ooi *et al.* [95]. D'autres études montrent que cette amplification se retrouve dans moins de 1% des cas [96]. Par conséquent, l'association entre l'amplification d'ESR1 et la résistance à l'hormonothérapie, ainsi que son rôle clinique, n'est pas encore bien définie [49,60].

Les modifications épigénétiques, entraînant une modification réversible de l'expression des gènes sans changer la séquence d'ADN, peuvent également altérer l'activité d'ER. Sous la pression de sélection des thérapies hormonales, la reprogrammation épigénétique favorise l'hétérogénéité phénotypique et l'expansion

des clones résistants aux traitements endocriniens [97]. Par exemple, un niveau plus élevé de l'expression de KDM5B, un membre de la famille KDM5 histone H3 lysine 4 déméthylase, est associé à une hétérogénéité transcriptomique plus élevée et à un mauvais pronostic dans les tumeurs du sein ER-positives [98].

1.8. Akt et son rôle dans le CS

1.8.1. L'importance de la signalisation PI3K/Akt/mTOR dans le CS

La voie PI3K/Akt/mTOR est une voie de signalisation impliquée dans la croissance, la prolifération, la survie, la motilité et le métabolisme cellulaire, ainsi que dans la régulation de la réponse immunitaire [99,100]. L'hyperactivation de cette voie est impliquée dans la carcinogénèse du CS ER-positif et dans la résistance aux thérapies endocriniennes. Les altérations de gènes codant pour plusieurs nœuds de la voie PI3K/Akt/mTOR sont fréquemment trouvées dans le CS ER-positif. Par conséquent, cette voie peut avoir un rôle de cible thérapeutique potentielle, ainsi que de valeur pronostique et diagnostique chez les patientes atteintes d'un CS [101]. Plusieurs molécules ciblant des protéines impliquées dans cette voie sont actuellement étudiées, seules ou en combinaison avec d'autres traitements, pour traiter des cas de CS résistants à l'hormonothérapie.

Dans notre étude, nous nous intéressons à Akt, la protéine centrale de cette signalisation qui sera discutée en détails dans les parties suivantes.

1.8.2. Structure d'Akt et de ses isoformes

Akt (RAC-alpha serine/threonine-protein kinase), aussi connu sous le nom PKB (protein kinase B) est une sérine-thréonine kinase qui possède trois isoformes différentes codées par trois gènes différents : Akt1, Akt2 et Akt3 [102].

Les isoformes d'Akt ont une structure conservée comprenant trois domaines : PHD (Pleckstrin homology domain), CD (catalytic domain) et HM (hydrophobic C-terminal regulatory motif) [103] (Figure 9). Le domaine PHD situé à l'extrémité N-terminale d'Akt interagit avec les lipides membranaires pour faciliter la

reconnaissance d'Akt par les kinases en amont et la translocation membranaire [104]. Le domaine CD, la région catalytique centrale de la protéine, contient un résidu thréonine qui doit être phosphorylé pour l'activation d'Akt [104]. Et enfin le domaine C-terminale HM d'Akt contient un résidu de sérine conservé et requis pour la phosphorylation et l'activation de la kinase [104].

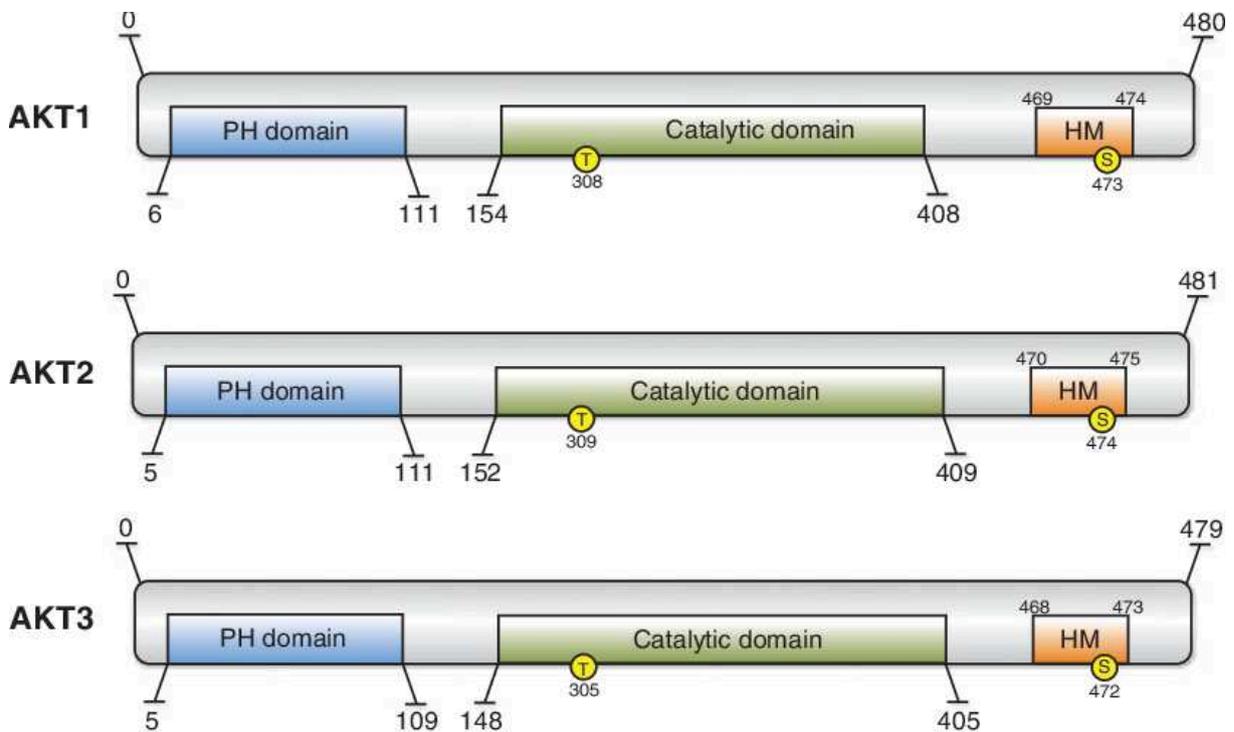


Figure 9. Structure des isoformes d'Akt. Représentation schématique de chaque isoforme d'Akt avec ses trois domaines : PH domain (Pleckstrin homology domain), CD (catalytic domain) et HM (hydrophobic C-terminal regulatory motif). Les emplacements des principaux résidus phosphorylés (soit T pour la thréonine ou S pour la sérine) sont également indiqués. Figure tirée de la publication de Fabi *et al.* [103]

Akt1 est principalement impliquée dans la régulation de la croissance et de la division cellulaire et est la plus associée au cancer. Akt2 joue un rôle important dans l'énergie cellulaire et le métabolisme. Akt3, l'isoforme la moins étudiée, a été proposée comme critique pour le développement du cerveau et la viabilité des cellules de gliome malin [105].

1.8.3. Mécanisme d'activation et voie de signalisation d'Akt

Le principal événement qui déclenche l'activation d'Akt est la liaison de ligands, tels que les facteurs de croissance, à leurs récepteurs membranaires [106,107]. La

liaison d'un facteur de croissance au RTK (receptor tyrosine kinase) conduit à l'activation de PI3K, qui recrute ensuite Akt à la membrane plasmique en liant le PH domaine aux lipides membranaires. Akt subit alors un changement de conformation qui conduira à la phosphorylation du résidu thréonine par PDK1 (phosphoinositide-dependent kinase-1) et du résidu sérine par mTORC2 (target of rapamycin complex 2). Une fois phosphorylées, les Akts activées se translocalisent à divers endroits intracellulaires, où ils phosphorylent et modulent la fonction de nombreux substrats impliqués dans la survie et la croissance cellulaire (Figure 10).

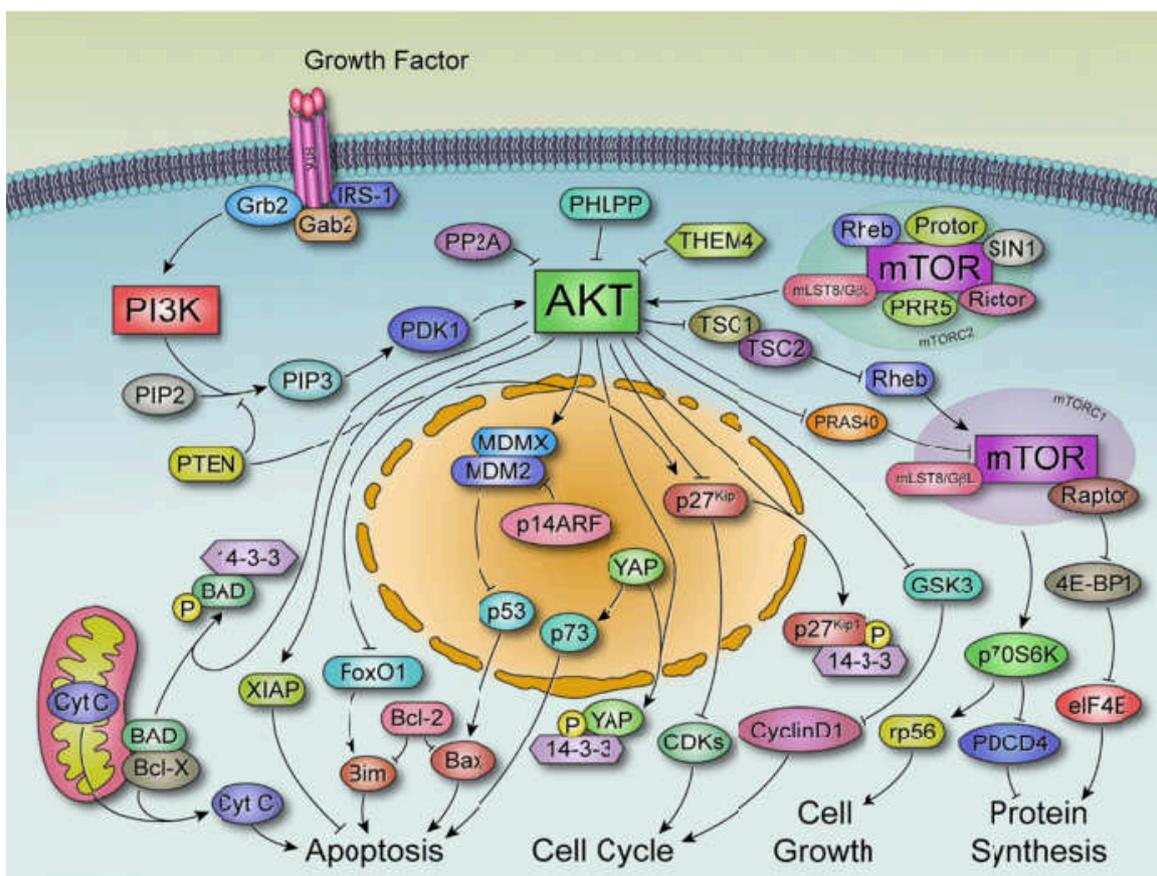


Figure 10. Représentation de la voie Akt et des principales cibles moléculaires impliquées dans la signalisation d'Akt. Figure tirée de la publication de Shariati *et al.* [105]

1.8.3.a. Akt/mTOR

L'une des principales cibles en aval de la signalisation d'Akt est la protéine mTOR. Une fois activée, mTOR phosphoryle la protéine p70S6K (Ribosomal protein S6 kinase) et la protéine 4E-BP1 (eIF4E binding protein-1) pour favoriser la synthèse

des protéines [108] (Figure 10). mTOR possède un rôle de régulateur de la traduction des protéines, du métabolisme, de la croissance et de la prolifération cellulaire. Cette fonction est fréquemment perturbée dans certains cancers dont le CS. La protéine mTOR est ainsi une cible importante dans le traitement des cancers [109].

1.8.3.b. Akt/Foxo/FOXM1

Akt est aussi impliquée dans la régulation de nombreux facteurs de transcription comme les FOXOs (forkhead box class O) dont FOXO3a (Figure 11) [110]. Akt phosphorylée limite la translocation nucléaire de FOXO3a en induisant sa translocation cytoplasmique et sa dégradation [111]. La translocation nucléaire de FOXO3a inhibe la transcription de FOXM1 (forkhead box protein M1). De plus, les gènes activés par FOXM1 sont réprimés par FOXO3a. Par conséquent, l'activation d'Akt favorise l'activation de FOXM1.

Alors que les FOXOs sont des suppresseurs de tumeurs, FOXM1 se comporte comme un oncogène classique. Conformément à son rôle dans la prolifération, la surexpression de FOXM1 a été identifiée dans de nombreux types de cancers, tels que les cancers du foie, de la prostate, du sein, du poumon et du côlon. En plus de la prolifération cellulaire, FOXM1 contrôle et influence d'autres processus biologiques, y compris la différenciation cellulaire, l'apoptose, l'angiogenèse, la sénescence, l'homéostasie tissulaire et la réparation des dommages à l'ADN [111].

Dans le CS, FOXM1 régule de nombreux « hallmarks » du cancer, y compris la prolifération, la mitose, l'EMT, l'invasion et les métastases [112]. Les études précliniques et cliniques ont montré que la surexpression de FOXM1 était corrélée à la progression de CS et à la résistance aux thérapies et entraîne ainsi un mauvais pronostic [112,113]. Ainsi, FOXM1 est une cible importante pour développer des traitements du CS, particulièrement pour les CS résistants aux traitements.

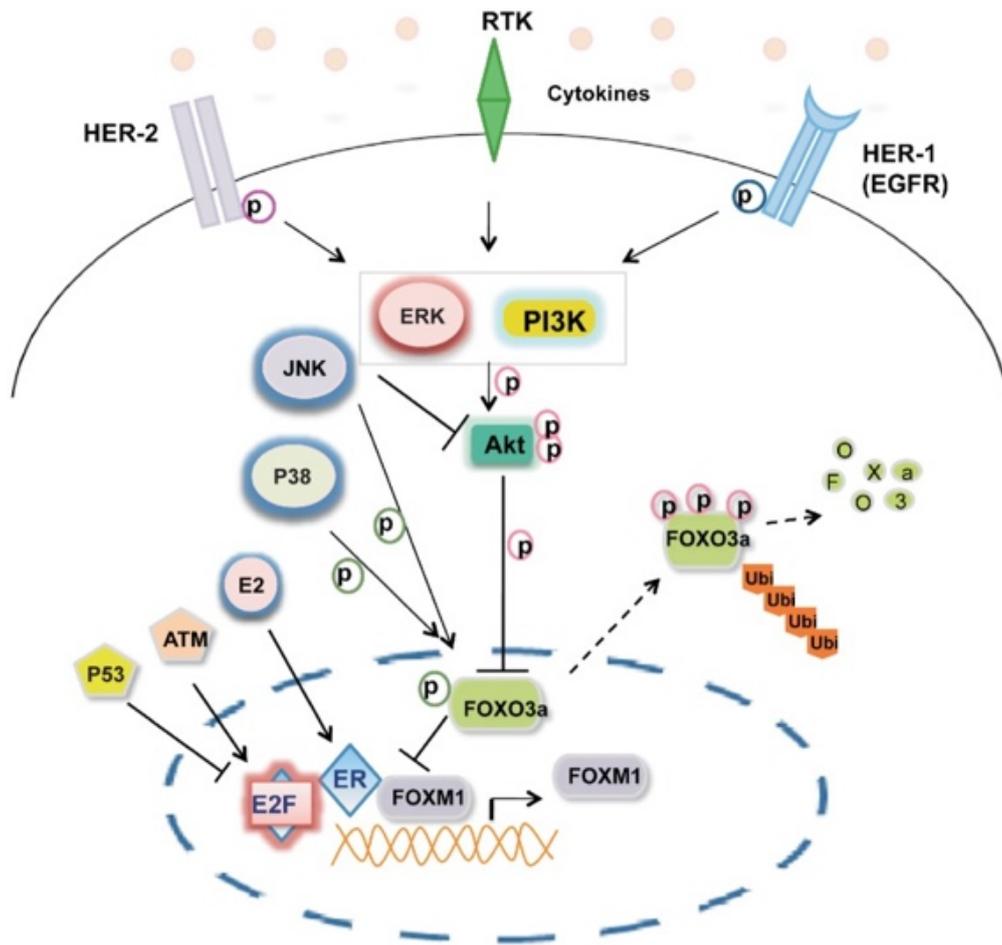


Figure 11. Cascade de signalisation PI3K/Akt/FOXO3a/FOXM1. Akt inhibe l'activité de FOXO3a qui antagonise la transcription de FOXM1. Par conséquent, l'activation d'Akt favorise la transcription de FOXM1, qui contrôle les processus liés au cancer, y compris la prolifération, la survie, la résistance aux médicaments, l'angiogenèse, la migration et la réparation des dommages à l'ADN. EGFR: human epidermal growth factor receptor; HER-2: human epidermal growth factor receptor 2; ERK: extracellular signal-regulated kinase; JNK: c-Jun N-terminal kinase; P38: P38 mitogen-activated protein kinase; P53: tumor suppressor protein 53; E2F: E2F transcription factor; ER: estrogen receptor; E2: estradiol; ATM: ataxia telangiectasia mutated protein; Ub: ubiquitination; P: phosphorylation. Figure tirée de la publication de Gomes *et al.* [111].

1.8.4. Hyperactivation d'Akt dans le CS

L'hyperactivation d'Akt peut favoriser la résistance aux thérapies endocriniennes de certaines patientes atteintes d'un CS. Chez ces patientes, une corrélation inverse a été établie entre l'activation d'Akt et une réponse partielle aux traitements [114,115]. La mutation la plus fréquente est E17K-Akt1, représentant 4 à 8% de tous les cancers du sein [116]. Cette mutation augmente l'affinité du domaine PH pour les lipides, entraînant la localisation constitutive d'Akt dans la membrane

cellulaire [117]. En clinique, la mutation E17K-Akt1 s'avère être un puissant biomarqueur prédictif de la réponse à la monothérapie AZD5363 [118].

1.8.5. Traitements anti-cancéreux ciblant Akt

L'inhibition d'Akt en tant que composant central de la signalisation PI3K/Akt est depuis longtemps une approche thérapeutique intéressante dans le cancer. Plusieurs composés ciblant Akt sont au stade de l'essai clinique (Tableau 1). Il existe deux classes d'inhibiteurs d'Akt: les inhibiteurs compétitifs de l'ATP et les inhibiteurs allostériques. Les inhibiteurs compétitifs de l'ATP se lient au site actif d'Akt, bloquant la liaison à l'ATP; tandis que les inhibiteurs allostériques se lient au domaine PH empêchant la phosphorylation et l'activation d'Akt [105].

Tableau 1. Médicaments ciblant Akt dans les essais cliniques.

Inhibiteur	Classe	Cible	Statut
Ipatasertib (GDC-0068)	ATP-compétitif	Akt1, 2 et 3	Essai clinique phase III
Capivasertib (AZD5363)	ATP-compétitif	Akt1, 2 et 3	Essai clinique phase II
ARQ751 et ARQ092	Allostérique	Akt1, 2 et 3	Essai clinique phase I
Afuresertib (GSK2110183) et Uprosertib (GSK2141795)	ATP-compétitif	Akt1, 2 et 3	Essai clinique phase II
MK-2206	Allostérique	Akt1 et 2	Essai clinique phase II
Perifosine	Allostérique	Akt1, 2 et 3	Essai clinique phase I
BAY1125976	Allostérique	Akt1 et 2	Essai clinique phase I
Triciribine	Allostérique	Akt1, 2 et 3	Essai clinique phase I
TAS-117	Allostérique	Akt1, 2 et 3	Essai clinique phase II
LY2780301	ATP-compétitif	Akt1, 2, 3 et p70S6K	Essai clinique phase I
MSC2363318A	ATP-compétitif	Akt 1, 3 et p70S6K	Essai clinique phase I
Cenisertib (763/AS703569)	ATP-compétitif	Akt, FLT3, VEGFR2, LYN, BTK et KIT	Essai clinique phase I

Traduit à partir du tableau issu de la publication de Shariati *et al.* [105]

Malgré le développement d'un grand nombre de composés avec des résultats prometteurs pour l'inhibition d'Akt, aucun des inhibiteurs d'Akt n'a été approuvé à ce jour pour une utilisation clinique. Nombre d'entre eux ont montré une efficacité clinique limitée en tant qu'agent unique et ont des limites en raison de leur toxicité. La fonction cellulaire complexe d'Akt et l'activation de la signalisation par rétroaction potentielle limitent l'efficacité des inhibiteurs d'Akt en tant qu'agent unique. Ainsi, le développement de molécules comme les molécules naturelles qui

peuvent agir sur plusieurs sites dans la voie d'Akt et qui ont une faible toxicité pourraient être une bonne stratégie. Une autre stratégie importante est de combiner des inhibiteurs d'Akt avec les traitements existants pour augmenter leur efficacité.

1.9. Voie d'AhR (Aryl hydrocarbon receptor) et son rôle dans le cancer du sein

1.9.1. Histoire d'AhR

Dans les années 70-80, après plusieurs incidents industriels tels que l'incident de Seveso en 1976, une maladie cutanée sévère appelée chloracné a été observée chez les travailleurs et les populations exposés [119,120]. Les produits chimiques suspectés ont été testés sur l'oreille du lapin et la 2,3,7,8-tétrachlorodibenzo-p-dioxine (TCDD) a été identifiée comme l'agent responsable le plus puissant [121]. Le « Aryl hydrocarbon receptor » (AhR) ou récepteur aux hydrocarbures aromatiques est découvert par Poland en 1976 lors de ses travaux pour comprendre les mécanismes responsables de la toxicité de la TCDD [122]. En 1992, deux groupes de scientifiques ont identifié indépendamment AhR comme facteur de transcription activé par le ligand de la famille Per-Arnt-Sim [123,124]. L'année suivante, l'ADNc codant AhR humain a été identifié [125]. Pendant longtemps, AhR est surtout considéré comme un médiateur des composés chimiques et environnementaux et utilisé pour étudier la toxicité de ces composés. Plusieurs xénobiotiques, dont les hydrocarbures aromatiques halogénés, ont été identifiés comme ligands d'AhR. Cependant, à travers ces études, nous nous sommes rendus compte qu'AhR n'est pas seulement un médiateur des toxiques mais que ce récepteur a aussi son propre rôle dans les fonctions physiologiques [121]. Actuellement, AhR est envisagé comme une cible thérapeutique potentielle pour plusieurs maladies dont le cancer.

1.9.2. La structure et la signalisation d'AhR

AhR fait partie de la famille des facteurs de transcription de basic helix-loop-helix / Per-Arnt-Sim [123,124] (Figure 12). Il est composé d'un domaine N-terminal bHLH (basic helix-loop-helix), de deux domaines Per-Arnt-Sim (également connus

sous le nom de PAS) A et B, et d'un domaine de transactivation C - terminal (TAD). Le domaine bHLH N-terminal est composé d'une séquence de quatre à six acides aminés basiques, nécessaires à la dimérisation et à la liaison à l'ADN. Chacun des deux domaines PAS est composé d'environ 110 acides aminés et sont nécessaires pour l'hétérodimérisation avec l'ARNT. PAS-A empêche la dimérisation avec des protéines bHLH ne contenant pas de PAS, régulant ainsi l'activité du domaine bHLH. Cela améliore la stabilité du complexe AhR-ARNT et renforce la liaison à l'ADN. Le domaine PAS-B est impliqué dans l'hétérodimérisation avec l'ARNT et contient le LBD [126].

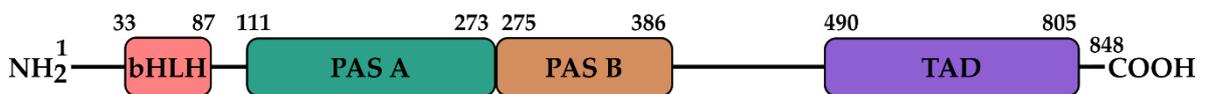


Figure 12. Structure de l'aryl hydrocarbon receptor montrant (de gauche à droite) the basic helix-loop-helix (bHLH), period -aryl hydrocarbon receptor nuclear translocator-single minded domain A (PAS-A), PAS-B and transactivation domain (TAD).

AhR est un facteur de transcription activé par la fixation du ligand (Figure 13). En absence de ligand, AhR est situé dans le cytoplasme cellulaire, il y est complexé avec un dimère de Hsp90 (heat shock protein 90), le p23 (prostaglandin E synthase 3) et la XAP-2 (X-associated protein 2), connue sous le nom d'AIP (aryl hydrocarbon receptor-interacting protein). La liaison du ligand entraîne un changement de conformation qui dévoile les signaux de localisation nucléaire d'AhR et une relocalisation nucléaire. Une dissociation du complexe se produit et AhR forme un hétérodimère avec l'ARNT. Une fois l'hétérodimère formé, AhR peut se lier à des XREs (xenobiotic response elements). Suite à cette liaison, l'activation transcriptionnelle de certains gènes qui contiennent des XREs dans leur séquence promotrice peut se produire; ceux-ci incluent les cytochromes P450 1A1, 1A2 et 1B1 (CYP1A1, CYP1A2 et CYP1B1), ainsi que l'AhRR (aryl hydrocarbon receptor repressor). AhR sans ligand est ensuite libéré et transporté vers le cytosol [126,127].

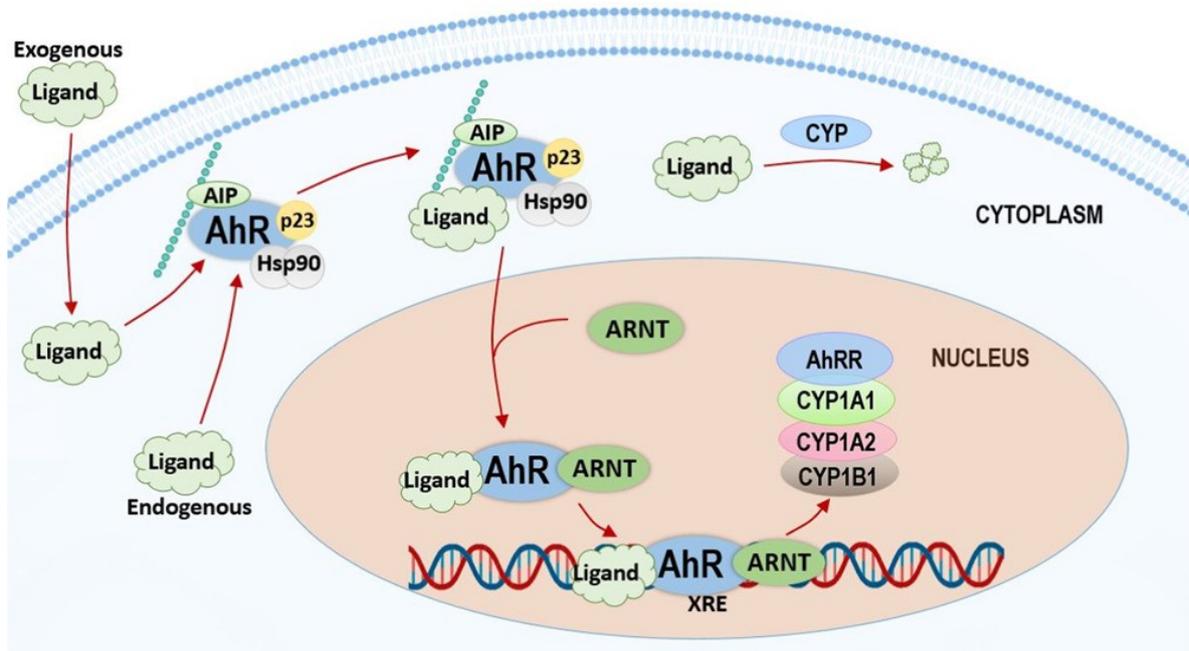


Figure 13. Voie de signalisation de l'aryl hydrocarbon receptor (AhR). AhR sans ligand est dans le cytosol, attaché à un filament d'actine dans le cadre d'un complexe avec deux molécules de Hsp90 (heat shock protein 90), la co-chaperone p23 et l'AIP (aryl hydrocarbon receptor-interacting protein). Lors de la liaison du ligand, une translocation dans le noyau cellulaire se produit, avant qu'AhR ne forme un hétérodimère avec ARNT (aryl hydrocarbon receptor nuclear translocator) et Hsp90 est libéré. L'hétérodimère se lie ensuite à des sections d'ADN appelées XRE (xenobiotic response element), entraînant l'activation transcriptionnelle de certains gènes tels que CYP1A1, CYP1A2, CYP1B1 et AhRR (aryl hydrocarbon receptor repressor). Schéma de Baker *et al.* [126]

1.9.3. Ligands d'AhR

AhR peut être activé par de nombreux composés synthétiques et naturels [128]. Les ligands d'AhR peuvent être exogènes ou endogènes et peuvent avoir une activité agoniste ou antagoniste sur le récepteur. Un résumé de ces ligands se trouve dans le tableau 2.

Tableau 2. Source, classification et exemples des ligands d’AhR

Activité	Source	Groupe	Exemples
Agoniste	Xénobiotiques	Hydrocarbures aromatiques halogénés	<ul style="list-style-type: none"> • 2, 3, 7, 8-tétrachlorodibenzo-p-dioxine • dibenzofuranes • biphényles
		Hydrocarbures polyaromatiques	<ul style="list-style-type: none"> • 3-méthylcholanthène • benzo (a) pyrène • benzanthracène • benzoflavone
		Médicaments	<ul style="list-style-type: none"> • tranilast • leflutamide • oméprazole, lansoprazole • raloxifène • sulindac • flutamide • nimodipine, mexilétine
	Alimentation	Flavonoïdes	<ul style="list-style-type: none"> • quercétine • galangin
		Indoles	<ul style="list-style-type: none"> • indole-3-carbinol • 3, 3-diindoylméthane • indolo [3, 2b] carbazole
	Endogène	Métabolites du tryptophane	<ul style="list-style-type: none"> • acide kynurénique • kynurénine • 6-formylindolo [3,2b] carbazole • sulfate d'indoxyle
		Autres	<ul style="list-style-type: none"> • indirubine, indigo • bilirubine • prostaglandine G2 • 7-cétocholestérol
	Micro-organismes		<ul style="list-style-type: none"> • 3-méthylindole • tryptanthrine • Acide 1, 4-dihydroxy-2-naphtoïque • malassezin
Antagoniste	Xénobiotique		<ul style="list-style-type: none"> • 6, 2, 4, -triméthoxyflavone • GNF351127 • CH-223191
	Alimentation		<ul style="list-style-type: none"> • resvératrol • génistéine
AhR sélective modulateur	Xénobiotiques		<ul style="list-style-type: none"> • SGA360 • 3, 4-diméthoxy-a-naphthoflavone • 6-méthoxy-1, 3, 8-trichlorodibenzofurane

Adapté, traduit et complété à partir du tableau issu la publication de Murray et al. [127]

1.9.4. Fonctions d’AhR

AhR est connu comme le médiateur de la toxicité des polluants environnementaux. Cependant, il est également impliqué dans la suppression de la

réponse immunitaire adaptative due à de nombreux contaminants environnementaux. De plus, il participe au bon fonctionnement du système reproducteur féminin à tous les stades, du développement fœtal jusqu'à l'âge adulte [129]. Il joue aussi un rôle comme modulateur de l'immunité antivirale [130]. De récentes études ont également montré les rôles cellulaires d'AhR dans les interactions fonctionnelles avec diverses voies de signalisation (y compris l'ER) et son rôle physiologique dans la régulation de nombreux processus cellulaires, y compris la prolifération cellulaire, le cycle cellulaire, la pluripotence et le caractère souche des cellules cancéreuses.

1.9.5. Rôles d'AhR dans le développement mammaire et dans la progression du CS

La glande mammaire est un tissu complexe qui subit de nombreux changements structurels et fonctionnels au cours des différents stades de la vie [131]. AhR peut jouer des rôles différents dans chacun de ces stades.

In utero, AhR est exprimé dès le 10^{ème} jour de gestation dans différents tissus embryonnaires de souris et son expression est spécifique à la fois du tissu et du stade de développement embryonnaire [132]. La suppression d'AhR chez la souris entraîne une diminution de 50% des bourgeons terminaux dans la glande mammaire [133]. L'exposition pendant la gestation et la lactation à la TCDD provoque chez les jeunes rattes un développement retardé de la glande mammaire bien que la capacité à se différencier en réponse aux œstrogènes soit conservée [134,135]. Cependant, il faut noter que ces réponses varient selon les moments d'exposition au cours de la gestation [136].

Concernant le rôle d'AhR dans le développement de tumeurs mammaires, l'activation d'AhR par la TCDD retarde la formation de tumeurs mammaires induites par le DMBA à la fois chez des souris multipares ou nullipares [137]. En revanche, l'exposition *in utero* augmente la sensibilité à la cancérogenèse [138]. Dans les cellules cancéreuses mammaires MDA-MB-231, la suppression d'AhR réduit la

croissance tumorale et les métastases, et rend ces cellules plus sensibles au paclitaxel [139]. L'expression d'AhR a été détectée à des niveaux élevés dans des tumeurs mammaires de stades avancés ou invasifs tandis que son expression est plus faible dans les premiers stades de la malignité ou dans le tissu mammaire non cancéreux [140,141]. De plus, AhR aurait un rôle pronostique. En effet, AhR est un marqueur de mauvais pronostic pour les patientes atteintes d'un CS ganglionnaire négatif [142].

1.9.6. Rôles d'AhR dans les métastases

Un nombre croissant d'études a montré qu'AhR joue un rôle dans les métastases. Il est montré qu'AhR a un rôle dans la modulation de l'adhésion et de la migration cellulaire [127]. La translocation nucléaire et l'activation d'AhR ont été observées lors de la perte de contact cellule-cellule par un mécanisme impliquant JNK (Jun N-terminal kinase) [143,144]. Cela indique que l'activation d'AhR peut entraîner des métastases. De plus, de nombreux facteurs pro-migratoires peuvent être modifiés par AhR [127]. Pourtant, il a été rapporté que l'activation d'AhR par ses ligands diminue l'expression de CXCR4, un promoteur des métastases [145,146].

Concernant la transition épithélio-mésenchymateuse (EMT), Tsai *et al.* ont montré que l'autophagie régulée par AhR affecte la progression de l'EMT des cellules cancéreuses dans le cancer du poumon [147]. Ils ont également montré qu'une augmentation du niveau d'AhR peut supprimer l'activité des histones désacétylases, ce qui conduit à inhiber la migration cellulaire dans le cancer gastrique [148].

1.9.7. Interaction entre les voies de signalisation d'AhR et d'ER

L'interaction entre ces deux voies de signalisation a été initialement observée dans des études *in vivo* : les tumeurs du sein et de l'utérus étaient, par exemple, moins fréquentes chez des rattes exposées de façon chronique à la TCDD [149]. Ces observations peuvent être expliquées par les concentrations circulantes d'œstrogène

significativement plus faibles chez les rattes exposées à la TCDD que chez les témoins [150] en raison de l'induction de CYP1A1 et CYP1B1 qui métabolisent les œstrogènes [151,152]. Les études qui suivent suggèrent que l'activation constitutive d'AhR, par exemple par une mutation, est œstrogénique tandis que l'activation d'AhR par un ligand semble avoir des effets anti-œstrogéniques [129].

Quatre compléments alimentaires à base de phytoestrogènes (la génistéine, la daidzéine, le S-équol du soja, et le liquiritigénine de la racine de réglisse) agissent via ER α et AhR; leur activité sur AhR entre en compétition avec leur activité sur ER α [153].

1.9.8. Effets des ligands d'AhR sur le CS

AhR est devenu une cible de plus en plus attractive pour trouver de nouveaux médicaments contre le cancer, notamment le CS [126,129,154]. Ainsi plusieurs ligands naturels et synthétiques d'AhR ont montré des effets anticancéreux contre le CS en modulant la signalisation d'AhR. Ces composés et leurs mécanismes d'action sont résumés dans le tableau 3.

Tableau 3. Mécanismes d'action des ligands d'AhR dans le cancer du sein

Ligands	Mécanisme d'action	Références
<i>Agonistes</i>		
Flavipine	Inhibition de la croissance et de l'adhésion des cellules MDA-MB-231 et T47D en supprimant l'expression des gènes BCL2 et ITGA4 Inhibition de la migration et de l'invasion de ces lignées cellulaires et suppression du facteur prométastatique SOX4 en induisant le cluster miR-212/132	[155]
Aminoflavone	Inhibition de la croissance des cellules du cancer du sein <i>in vitro</i> et <i>in vivo</i> via la voie de signalisation d'AhR Inhibition de la prolifération des cellules résistantes au tamoxifène en supprimant la signalisation α 6-intégrine-Src-Akt	[156–159]
β -Naphthoflavone	Induction de l'arrêt du cycle cellulaire dans la phase G0 / G1, conduisant à un phénotype de type sénescence, par régulation AhR-dépendante de la signalisation PI3K / AKT et MAPK / ERK dans les cellules MCF-7 mais pas les cellules MDA-MB-231	[160]
2-(4-amino-3-méthylphenyl)-5-fluorobenzothiazole (5F 203)	Induction de dommages à l'ADN par le stress oxydatif via la voie AhR dans de plusieurs lignées cellulaires de cancer du sein Induction de l'expression de la cytoglobine et inhibition de la croissance des cellules MDA-MB-468 et des tumeurs xéno greffées de MDA-MB-468	[161,162]
5,6,7,8-tétrahydrocinnolin-5-ol (NK150460)	Inhibition de la prolifération des cellules cancéreuses mammaires ER-positives et ER-négatives par l'activation d'AhR	[163]
(Z)-2-(3,4-dichlorophenyl)-3-(1H-pyrrol-2-yl)acrylonitrile (ANI-7)	Induction de dommages à l'ADN, arrêt du cycle cellulaire en phase S et mort cellulaire par détournement de la voie AhR et induction de CYP1A1	[164]
Raloxifène	Induction de l'apoptose dans les cellules MDA-MB-231	[165]
Oméprazole	Diminution de l'invasion des cellules MDA-MB-231 <i>in vitro</i> et des métastases de ces cellules dans le poumon chez la souris en réduisant l'expression des gènes prométastatiques MMP-9 et CXCR4	[146]
Tranilast	Inhibition des cellules cancéreuses souches dans le cancer du sein triple négatif	[166]
<i>Antagonistes</i>		
Epigallocatechin-3 gallate (EGCG)	Inversement EMT dans les cellules tumorales mammaires traitées au DMBA	[167]
Génistéine	Sensibilité du cancer du sein triple négatif à la thérapie anti-œstrogène en prévenant et en inversant l'hyperméthylation de BRCA1	[168]

2. Molécules phytochimiques

2.1. Intérêt des composés phytochimiques

Les maladies chroniques telles que les maladies cardiovasculaires, le diabète ainsi que les cancers sont actuellement responsables de plus de 70% de la mortalité mondiale d'après l'organisation mondiale de la santé (OMS) [169]. Il a été démontré que les fruits, les légumes et les céréales exercent non seulement un effet protecteur contre le développement de ces maladies mais aussi un effet thérapeutique [170]. Une étude épidémiologique menée au Japon montre que la consommation de jus de tomate non salé améliore la pression artérielle systolique et diastolique et le taux de LDL-C sérique chez les résidents japonais qui ont un risque de maladies cardiovasculaires [171]. D'autres études effectuées chez les animaux et chez l'homme prouvent que les myrtilles jouent un rôle préventif contre l'athérosclérose [172]. Le régime méditerranéen, qui est riche en légumes et fruits frais, huile d'olive et noix, est largement reconnu comme un modèle de « healthy eating », en diminuant le risque des maladies métaboliques et cardiovasculaires [173].

Le rôle bénéfique des fruits, des légumes, des céréales et d'autres plantes peut être principalement attribué aux composés phytochimiques qu'ils contiennent et qui sont définis comme des composés bioactifs non nutritifs [170]. Jusque-là, environ 10 000 substances phytochimiques ont été identifiées et un nombre important de ces substances est encore inconnu. Les composés phytochimiques identifiés comprennent les tanins, les flavones, les triterpénoïdes, les stéroïdes, les saponines et les alcaloïdes. Le rôle protecteur ou thérapeutique des composés phytochimiques peut être associé à leur activité antioxydante, anti-inflammatoire et anti-cancéreuse [170].

Parmi les composés phytochimiques, plusieurs molécules ont montré scientifiquement leur effet bénéfique sur le CS. Voici quelques exemples résumés dans le tableau 4.

Tableau 4. Exemples de composés phytochimiques ayant des effets sur le cancer du sein

Molécules	Sources	Effets sur le cancer du sein	Références
Curcumine	Curcuma	Inhibe la croissance tumorale, l'angiogenèse et la migration, provoque l'apoptose, modifie les marqueurs épigénétiques	[174-177]
Resvératrol	Peau de raisin, noix, fruits rouges	Inhibe la croissance tumorale, provoque l'apoptose, inhibe l'angiogenèse	[178,179]
Quercétine	Câpre, livèche, piment	Inverse la résistance à la chimiothérapie, provoque l'apoptose et l'arrêt de la prolifération cellulaire, inhibe l'angiogenèse	[180-182]
Sulforaphane	Légumes crucifères	Inhibe la croissance tumorale, provoque l'arrêt de la prolifération cellulaire et l'apoptose, modifie les marqueurs épigénétiques	[183-187]

2.2. SERMs (*Selective estrogen receptor modulators*) parmi les molécules phytochimiques

Les SERMs sont des agents synthétiques non stéroïdiens, qui ont des activités agonistiques ou antagonistiques d'ER dans différents tissus, probablement en raison des changements de conformation du récepteur associé à la liaison du SERM et de l'effet consécutif sur la transcription [188]. Ils sont en fait des ligands d'ER qui, dans certains tissus, agissent comme les œstrogènes, mais bloquent l'action des œstrogènes dans d'autres par une inhibition compétitive de la liaison des œstrogènes à ER (Figure 14) [188]. Par exemple, le tamoxifène, le SERM le plus couramment utilisé, est connu pour avoir des effets antagonistes dans le tissu mammaire, alors qu'il peut avoir des effets agonistes ou agonistes partiels au niveau de l'utérus, de l'os et des vaisseaux [189]. Des études ont montré que des SERMs ont la capacité d'induire des changements structurels distincts de l'ER qui pourrait influencer sa capacité à interagir avec des coactivateurs ou des corépresseurs, qui sont impliqués dans la régulation de la transcription du gène cible [188]. En plus, différents ligands peuvent avoir des affinités différentes vers ER α ou ER β [188]. Par conséquent, l'action biologique résultante dans un tissu spécifique peut varier selon le type d'ER exprimé, de cofacteurs et de ligands conduisant à une activité agonistique ou antagonistique [188,190]. Comme évoqué dans la partie 1.6.2.a, les SERMs tels que

le tamoxifène et le raloxifène font partie des traitements endocriens contre le CS. En comparaison avec de purs antagonistes d'ER comme les SERDs, les SERMs ont un meilleur rapport bénéfices/ effets secondaires grâce à leurs effets sur des tissus cibles sélectionnés. Le tamoxifène antagonise ER dans le tissu mammaire ; par conséquent, ce SERM inhibe la prolifération des cancers mammaires ER-positifs. En même temps, le tamoxifène a un effet agonistique sur l'activité d'ER dans les tissus osseux, ce qui pourrait protéger les femmes ménopausées de l'ostéoporose. Cependant, cette molécule a un effet agonistique sur l'activité d'ER dans l'utérus, ce qui entraîne une augmentation du risque de cancer dans cet organe. C'est pourquoi, bien que le tamoxifène soit le SERM le plus utilisé et que beaucoup de femmes en bénéficient, la recherche pour trouver de nouveaux SERMs, encore plus ciblés sur les sites thérapeutiques désirés, est une nécessité.

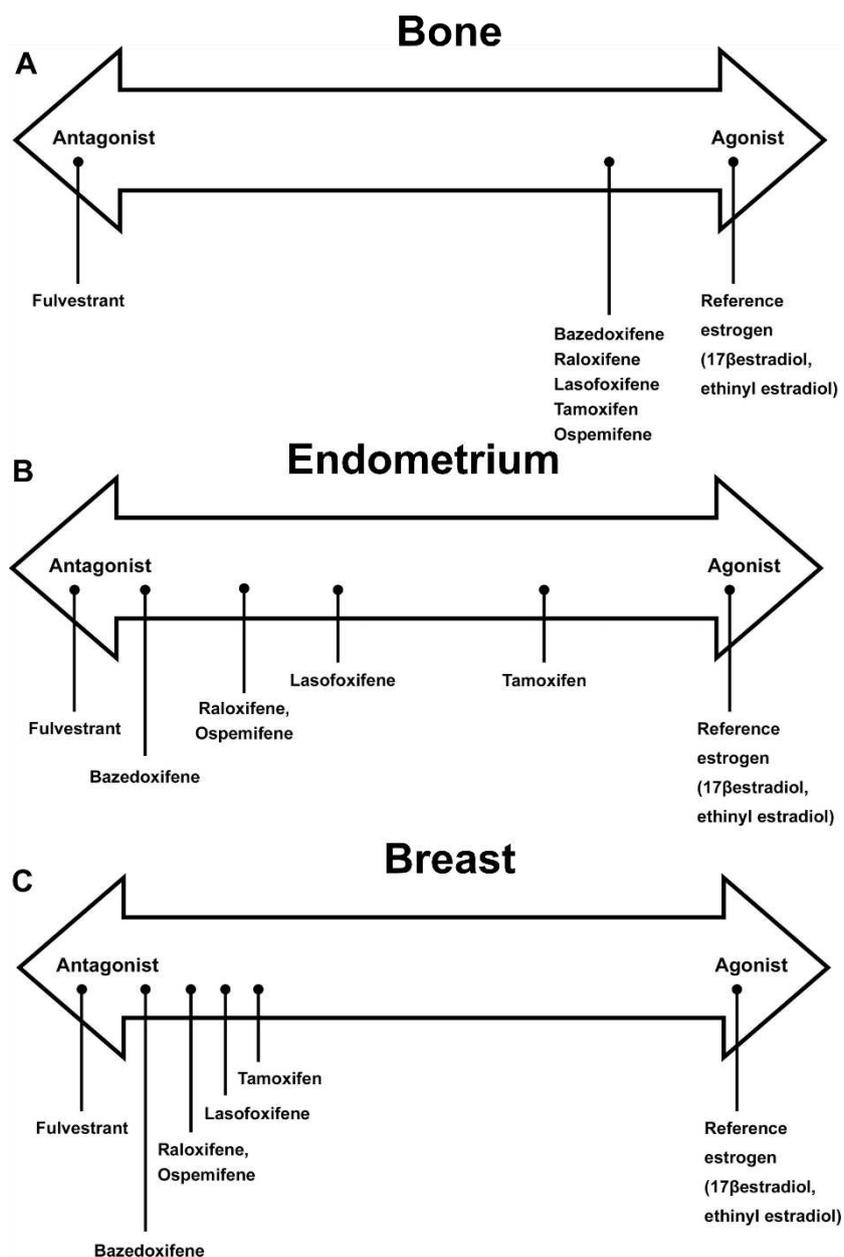


Figure 14. Activité des SERMs dans différents tissus ciblés. (A) Dans les os ; (B) dans l'endomètre ; (C) dans le sein. Figure tirée de la publication de Komm *et al.* [191]

Dans cette démarche, les molécules naturelles, surtout les phytoestrogènes constituent une piste intéressante. Les phytoestrogènes sont des dérivés végétaux qui présentent une similitude structurelle avec E2 et agissent sur ER. Les phytoestrogènes comme 8-prényl génistéine et glabridine ont montré les propriétés SERMs [192]. Elles sont considérées comme les phyto-SERMs.

Intéressée par les propriétés de SERMs et par les effets bénéfiques des molécules naturelles sur le CS, notre équipe a fait un criblage de l'effet de plusieurs molécules

issues de végétaux sur le CS. Parmi les molécules étudiées, nous avons montré que les glycéollins et l'apigénine présentent des bénéfices potentiels pour la santé humaine [193–195]. Nous avons donc choisi d'étudier ces deux composés au cours de cette thèse.

2.3. *Glyceollins*

Synthèse de la publication N°1: "An Update on the Effects of Glyceollins on Human Health: Possible Anticancer Effects and Underlying Mechanisms"

Les glyceollins, de la famille des phytoalexines, sont les composés bioactifs les plus importants présents dans la graine de soja suite à l'exposition à certains champignons et à certains éliciteurs abiotiques. Ils sont synthétisés *de novo* à partir de l'isoflavone de soja, la daidzéine. Les glycéollins agissent comme un système de protection des plantes grâce à leurs actions anti-bactériennes, anti-fongiques et anti-nématodes. Récemment, ils sont devenus de plus en plus attractifs en raison de leurs activités anti-cancéreuses, anti-oxydantes et anti-inflammatoires. Ainsi, ils ont montré des effets préventifs ou thérapeutiques sur des maladies chroniques telles que le cancer, les maladies cardiovasculaires, le diabète ou les maladies neurodégénératives.

Dans cette revue de synthèse sur les glycéollins, l'intérêt de ces molécules bioactives sur la santé humaine a été décrit dans l'introduction générale. Puis, la synthèse, la structure, le métabolisme et la pharmacocinétique de ces molécules ont été discutés. D'autres parties de cette revue sont consacrées aux effets des glycéollins sur le cancer, à la fois œstrogène-dépendant et œstrogène-indépendant. Nous avons également discuté de leurs effets sur des maladies non cancéreuses, notamment, l'ostéoporose, les troubles du métabolisme du glucose et des lipides, le stress oxydatif, les maladies neurodégénératives, l'inflammation, les maladies cardiovasculaires et la mélanogénèse. La revue se termine par une conclusion résumant les effets des glycéollins et les stratégies pour étudier ces molécules par la suite.

PUBLICATION N°1 « An Update on the Effects of Glyceollins on Human Health: Possible Anticancer Effects and Underlying Mechanisms »

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Review

An Update on the Effects of Glyceollins on Human Health: Possible Anticancer Effects and Underlying Mechanisms

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Abstract: Biologically active plant-based compounds, commonly referred to as phytochemicals, can influence the expression and function of various receptors and transcription factors or signaling pathways that play vital roles in cellular functions and are then involved in human health and diseases. Thus, phytochemicals may have a great potential to prevent and treat chronic diseases. Glyceollins, a group of phytoalexins that are isolated from soybeans, have attracted attention because they exert numerous effects on human functions and diseases, notably anticancer effects. In this review, we have presented an update on the effects of glyceollins in relation to their potential beneficial roles in human health. Despite a growing number of studies suggesting that this new family of phytochemicals can be involved in critical cellular pathways, such as estrogen receptor, protein kinase, and lipid kinase signaling pathways, future investigations will be needed to better understand their molecular mechanisms and their specific significance in biomedical applications.

Keywords: glyceollins; phytochemicals; dietary compounds; signaling pathways; estrogen receptor; breast cancer; human health

1. Introduction

Thousands of bioactive molecules, among phytochemicals, naturally occur in plants after secondary metabolism. Out of these compounds, polyphenols represent the most important family, including more than 500 identified compounds [1,2]. They are mainly composed of flavonoids, phenolic acids, stilbenes, and lignans, found in a wide variety of plant-based foods. Polyphenol concentrations in foods and quantities consumed differ considerably, leading to wide differences in the daily intake between polyphenol compounds [2]. Polyphenols have often been associated with a decreased incidence of several human diseases and have been used in traditional medicines and functional foods [3]. Epidemiological studies have shown that a high intake of polyphenolic phytochemicals in the diet can prevent many diseases, such as cancers, diabetes or inflammatory, cardiovascular, and neurodegenerative diseases [4–9]. However, these studies have some limitations because they do not always take into account the geographical diversity, sex, age, and region of residence of the subjects. In addition, an accurate evaluation of polyphenol intake, which appears to be essential in these studies, is not always completed [2,8]. Polyphenols are also used as food supplements and in pharmaceutical and cosmetic products [10–12]. One of the advantages of ingesting phytochemicals through the diet is that combinatory effects from these compounds can occur [9].

The phytoalexins, a group of polyphenolic compounds possessing strong antimicrobial and antifungal properties, are produced by plants as defense molecules against phytopathogens [13]. They are only present in very small quantities in healthy plants, but accumulate in large quantities following an attack by bacteria, fungi, or nematodes [14,15]. In addition to biotic agents, other stresses, such as high temperature, ultraviolet (UV) radiation, humidity, and dryness, can also induce the production of these compounds [14,15]. Phytoalexins are very diverse and have been found in various crops, such as rice [16], soybean [17], maize [18], barley [19], and banana [20]. Phytoalexins can be produced in numerous parts of the plant, such as the flowers, leaves, stems, seeds, and root tubers [19,21].

Glyceollins, a group of phytoalexins, are the most important bioactive compounds present in soybeans upon exposure to certain fungi and some abiotic elicitors, such as UV light, aluminum chloride, or methyl jasmonate [17,22]. Glyceollin I, II, and III (GI, GII, and GIII) are de novo synthesized from the soy isoflavone daidzein. In addition to their antibacterial, antifungal, and antinematode actions, glyceollins have recently received much attention because of their antiproliferative, antiestrogenic, anti-inflammatory, antioxidative, and anticholesterolemic activities. They may possess potential medicinal properties in humans, notably protective effects against hormone-dependent cancers, and metabolic and cardiac diseases (Figure 1) [23,24]. Accordingly, glyceollins are effectively able to inhibit proinflammatory cytokines by inhibiting the activation/phosphorylation of the transcription nuclear factor-kappa B (NF- κ B). They prevented the lipopolysaccharide (LPS)-induced expression of nitric oxide synthase (iNOS) and cyclo-oxygenase (COX)-2 in murine macrophage cell lines and were able to reduce 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced skin inflammation in mice. These natural compounds also inhibit hormone-dependent breast and ovarian cancer cell growth [25,26]. One of the mechanisms responsible for the antitumoral effect of glyceollins is their antiestrogenic action [25,27,28]. However, other antitumorigenic actions, whose mechanisms are not well understood, of glyceollins have also been reported. For instance, Lee et al. reported on an inhibitory effect of glyceollins on the kinase activity of the vascular endothelial growth factor (VEGF) receptor as well as its downstream signal transduction pathways involved in angiogenesis and tumor growth [29]. Likewise, an inhibitory effect on the expression of hypoxia inducible factor 1 (HIF-1)-target genes (such as VEGF) in cancer cells was described [30]. In addition, glyceollins were able to inhibit phosphatidylinositol 3-kinase/protein kinase B/mammalian target of rapamycin (PI3K/AKT/mTOR) pathways involved in the control of HIF-1 expression in solid tumor tissues. Moreover, a study by Carriere et al. showed that the epithelial-mesenchymal transition of breast cancer cells that are resistant to aromatase inhibitors can be blocked by GI [31]. This effect is partially mediated by the inhibition of zinc finger E-box binding homeobox 1 (ZEB1) expression.

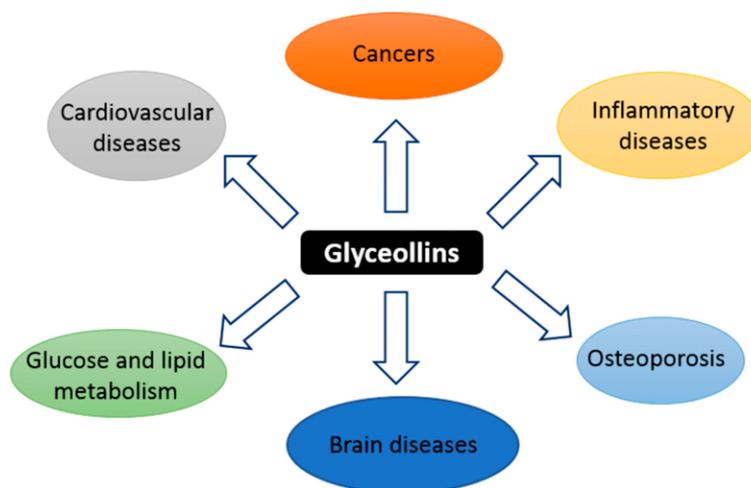


Figure 1. Schematic summary of the targets of glyceollins.

In summary, particular attention has recently been given to the effects of glyceollins on human health, including their chemo-preventive and antitumoral functions. A growing number of in vitro and in vivo studies have examined these effects [23,24]. In this review, we will first discuss the anticancer properties of glyceollins and their promising effects as new therapeutic agents or dietary supplements in breast and other cancer treatments and then briefly review the potential health benefits of these compounds in other noncancer diseases.

2. Synthesis and Structure

Previously described as hydroxyphaseollin in the early 70s [32,33], glyceollins belong to the family of phytoalexins, which are antimicrobial agents in plants. Glyceollins were characterized in soybeans after inoculation with the pathogen agent *Phytophthora sojae*. There are at least five glyceollins described to date that are produced via a complex metabolic pathway, even though GI, GII, and GIII are the most studied (Figure 2). Glyceollins are produced from the isoflavonoid branch of the phenylpropanoid pathway. Isoflavone daidzein is produced from phenylalanine and stored. Then, in the case of stress, daidzein will be metabolized into glyceollins. After multiple reduction steps, daidzein is transformed into (6aR, 11aR)-3,9 dihydroxypterocarpan. Pterocarpan is characterized by a pyrano-furano-benzene skeleton formed by the coupling of a B ring with the C ring of the isoflavonoid. Then, through the catalytic activity of CYP93A1 (also known as 3, 9 dihydroxypterocarpan 6a monooxygenase), the (6aR, 11aR)-3,9 dihydroxypterocarpan is metabolized into (-)-glycinol [34]. From glycinol, the metabolic pathway of glyceollins differs between glyceollin I and glyceollins II and III. In both cases, the next step consists of connecting a dimethylallyl group to glycinol through the activity of a trihydroxypterocarpan dimethylallyl transferase (also known as a prenyltransferase). For GII and GIII, dimethylallyl is connected to carbon 2, which leads to the production of (6aS, 11aS)-2-dimethylallyl-3, 6a, 9-trihydroxypterocarpan (also known as glyceollidin II) [35]. For GI, the connection of the dimethylallyl group occurs on carbon 4, which leads to the production of (6aS, 11aS)-4-dimethylallyl-3, 6a, 9-trihydroxypterocarpan (also known as glyceollidin I) [36]. In soybeans, approximately 77 prenyltransferases have been identified. Among them, five are induced in response to *Phytophthora sojae*, suggesting that they are involved in phytoalexin biosynthesis. A recent study [37] identified GmPT01 as a prenyltransferase that localized in the plastid and presented root-specific expression. These results suggested that GmPT01 could be involved in GI biosynthesis. The final step of glyceollin biosynthesis consists of the cyclization of the dimethylallyl group by glyceollin synthase. This enzyme belongs to the family of cytochrome P450 and was identified in 1988 [38]. To our knowledge, the specific enzymatic activity that leads to the different glyceollin isomers is not yet known.

Glyceollins are promising molecules in human health, and as such, it is important to decipher the molecular mechanism of the glyceollin biosynthesis pathway. For instance, in a recent study, the co-treatment of soybeans with silver nitrate (AgNO_3) and with the wall glucan elicitor of *Phytophthora sojae* was shown to specifically enhance the production of glyceollins by not only enhancing the expression of genes encoding specific enzymes, but also by inhibiting the degradation of glyceollins [39].

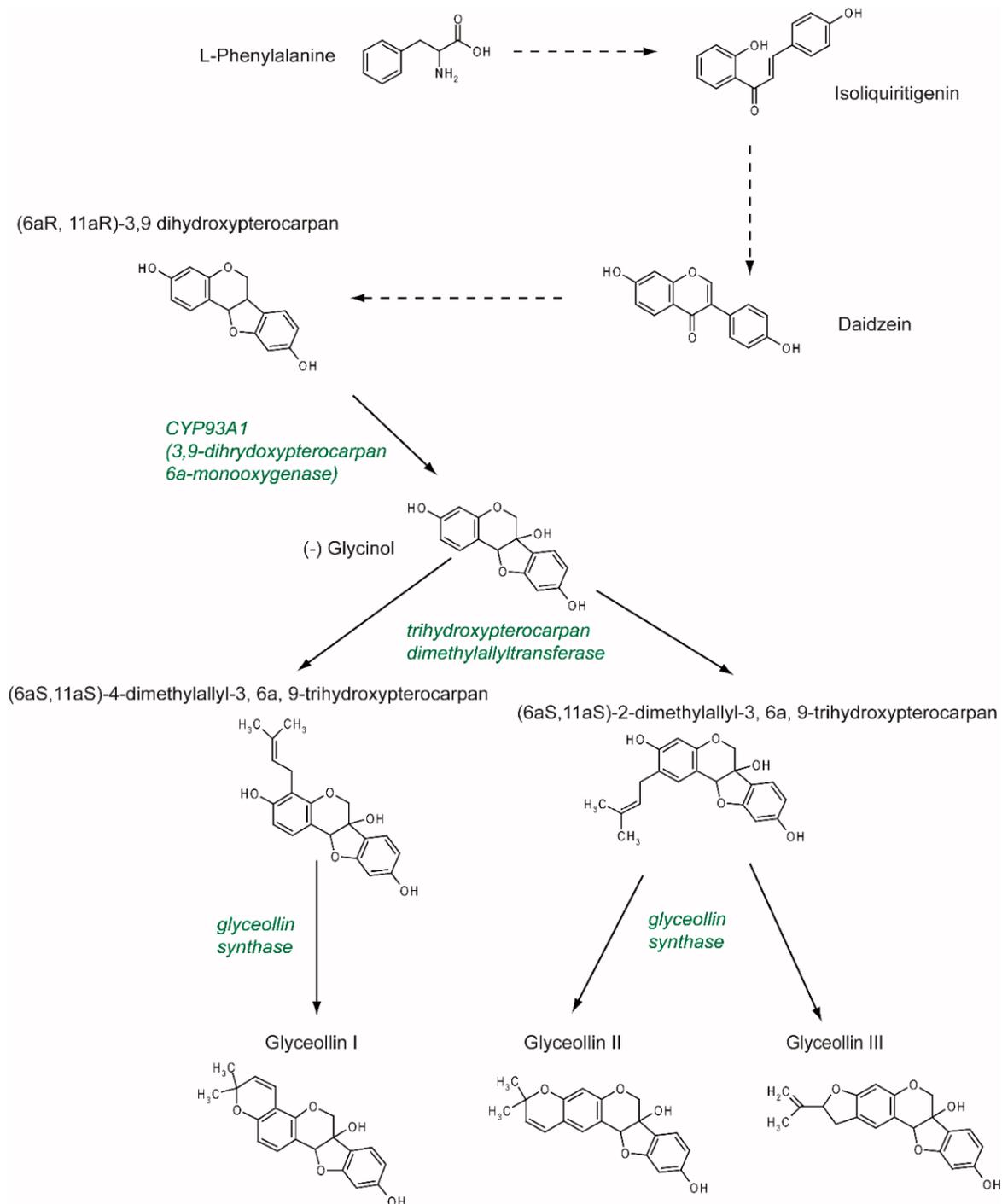


Figure 2. Glyceollin biosynthesis pathway. The isoflavone daidzein serves as the metabolic precursor for the rapid production of glyceollins. Daidzein is produced through the isoflavonoid branch of the phenylpropanoid pathway starting from L-phenylalanine. Under stress, daidzein is transformed into (6aR, 11aR)-3,9 dihydroxypterocarpan. This molecule is metabolized by CYP93A1 into (-) glycinol. Then, a dimethylallyl group is attached to the glycinol at position 2, which leads to the production of glyceollin II and III, or at position 4, which leads to the production of glyceollin I. These two steps are catalyzed by trihydroxypterocarpan dimethylallyl transferase and glyceollin synthase, respectively.

3. Metabolism and Pharmacokinetics

The beneficial antioxidant properties of isoflavones and their derivatives, phytoalexins, have generated a deep interest in their use as nutritional supplements. In terms of bioavailability and

pharmacodynamics, the most studied molecule among phytoalexins is resveratrol, which has a high commercial value. However, several recent studies have focused more specifically on the metabolism and pharmacokinetics of glyceollins (Table 1). For instance, in monkeys fed a glyceollin-enriched diet (9 mg/kg/day), the plasma level of glyceollins reached 134.2 nmol/L 4 h after oral administration and was completely undetectable 24 h later [40]. More interestingly, while glyceollins represented approximately 50% of the isoflavones present in the diet used in the previously mentioned study, they did not represent more than 11.6% of the isoflavones found in the plasma 4 h later, due to lower glyceollin absorption or a more rapid elimination [40].

The situation seems to be quite different in rodents, where bioavailability is low, but glyceollins appear to remain for a long time. When glyceollins were administered by oral gavage (30 or 90 mg/kg) to rats, glyceollins were found in the plasma starting at 20 min after administration, achieved a peak concentration (118.4 or 159 ng/mL) by 60 min and remained at a stable level until 4 h [41]. Such retention is not common for isoflavones, and a slow clearance may contribute to an accumulation of glyceollins and to a persistent exposure of rodents to glyceollins. However, the difference between the two doses tested (30 or 90 mg/kg) was not found at the plasma level [41]. This suggests that, in rodents, a dose of 30 mg/kg administered orally induces an almost maximal plasma concentration of glyceollins. This information, coupled with the notion of the persistence of glyceollins in rodents, explains the similar effects of different concentrations on the same model. Glyceollins have been shown to improve glucose homeostasis in diabetic rodents, whether orally delivered at a dose of 3 mg/kg [42] or at a dose of 30 mg/kg [41].

Thus, glyceollins have a conventional absorption rate, but their bioavailability is low. A better understanding of the destiny of ingested glyceollins was possible thanks to the identification of conjugated forms of glyceollins by ion scanning using liquid chromatography coupled online with electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) [43]. This technique thoroughly identifies the conjugated forms of glyceollins. It allowed the identification of glyceollin metabolites derived from the activity of phase I (hydroxylated forms) and phase II (sulfated forms, glutathione or glucuronide conjugates) metabolism in the blood, urine, and feces of orally exposed rats. The three unmetabolized isomers of glyceollins were detected in the feces, suggesting that glyceollins also undergo strong oxidation in the body [43,44]. Thus, first-pass intestinal and hepatic metabolism are barriers to the presence of glyceollins in systemic blood.

The low bioavailability of phytochemicals is notable due to their sulfation and glucuronidation by intestinal enzymes [45,46]. These conjugates, although they facilitate elimination and decrease bioavailability, may also be beneficial for the effects of some molecules. Thus, it has been shown that the sulfated form of resveratrol, which is a biologically inactive, but abundant form after oral intake, is captured by the cells that regenerate active resveratrol [47]. No data exist on such a mechanism with glyceollins.

Glyceollin conjugates have also been described *in vitro* in CACO-2 cells [48]. In this model, glyceollins (24 h of exposure of up to 100 μ M) also decreased the activity of the MRP2 and BCRP transporters (two major apically expressed efflux transporters in the intestine) without significant modification of their expression [49]. This effect can have important consequences on drug efficacy. It can be hypothesized that glyceollins could enhance the absorption of the chemical molecules used in therapy by inhibiting their apical excretion and possibly even enhancing their basolateral efflux, thereby increasing systemic delivery. This effect may reduce prescribed doses of drugs.

Finally, it should be noted that studies have recently been published on the effects of glyceollins on intestinal microbiota. In a mouse model, whose obesity was induced by the diet, the addition of glyceollins to the diet (65 μ g/mouse/day for 30 days) modified the microbiota to a less favorable profile for fat absorption, reduced the production of bile, and promoted the formation of short chain fatty acids [50]. All of these effects resulted in improved body composition.

Table 1. Bioavailability of glyceollins.

Animal Model	Treatment	Sample	Time	Method of Measure	Major Results	References
Female monkey (Macaca fascicularis)	Diet containing glyceollin mixture ¹ , 134 mg/day representing 50% of total isoflavonoids in the diet	Plasma	4 h and 24 h post administration (postad)	Liquid chromatographic-photodiode array mass spectrometric analysis	Plasma concentration of glyceollins: -4 h: 134.2 ± 34.6 nmol/L, representing only 11.6% of the plasma isoflavonoids level -24 h: Undetectable < 1 nmol/L	Wood et al. [40]
Male ZDSD/Peo rat	Glyceollin mixture, gavage, 30 and 90 mg/kg	Plasma	20, 60, 120 and 240 minutes (min) postad	HPLC-electrospray ionization-MS/MS	Plasma concentration of glyceollins: -20 min: Starts to be detectable -the next 220 min: Remains stable For dose 30 mg/kg: Ranges from 81.2 to 118.4 ng/mL For dose 60 mg/kg: Ranges from 118.2 to 159.0 ng/mL -60 min: Peak concentration	Boué et al. 2012 [41]
	Glyceollin mixture, gavage, 90 mg/kg/days for 2 weeks	Plasma, feces, and urine	Plasma: 3 h postad Feces: Once daily for 2 weeks Urine: 24 h collection postad a single dose	Precursor and product ion scanning using liquid chromatography coupled online with Electrospray ionization tandem mass spectrometry	-Rapidly absorption, glyceollins undergo phase I and phase II metabolism in the small intestine and the liver -Metabolites of glyceollins were identified in the plasma, the urine, and the feces Phase I conjugation: Epoxidation, hydroxylation ... Phase II conjugation: Sulfate and glucuronide conjugations ...	Quadri et al. 2013 [43], Quadri et al. 2014 [44]

¹ Glyceollin mixture contains glyceollin I, glyceollin II and glyceollin III.

4. Anticancer Effects

4.1. Estrogen-Dependent Effects

Like other phytoestrogens, glyceollins have a similar structure to that of the natural hormone, 17 β -estradiol (E2). Therefore, their effects in estrogen-dependent diseases have been the most investigated. Breast cancer is the most frequent type of cancer in women worldwide, with nearly 1.7 million new cases diagnosed in 2012 [51]. Among the different types of breast cancer, the most common is estrogen receptor (ER)-positive cancer, which represents approximately 75% of the diagnosed cases of breast cancer [52]. The ER belongs to the nuclear receptor superfamily and is divided into two subtypes, ER α and ER β (Figure 3). These receptors, which function primarily as ligand-activated transcription factors, regulate various cellular functions, such as proliferation, survival, differentiation, and apoptosis [53]. They are characterized by distinct domains comprising a conserved zinc finger DNA binding domain (DBD), a ligand binding domain (LBD), and two transactivation functions (AF). ERs typically alter the transcription of target genes by direct interaction with an estrogen-responsive element (ERE) and by recruiting transcription factors involved in chromatin remodeling [54]. However, many E2 target genes are devoid of an ERE in their promoter. In this case, ERs can modulate their transcription by establishing protein interactions with activator protein 1 (AP1) or specificity protein 1 (Sp1) transcription factors [55]. In addition, there are multiple levels of cross-talk between ERs and other intracellular pathways. Indeed, although ERs are mainly in the nucleus, a small amount of ERs can be present in the cytosol or near the plasma membrane. They are capable of interacting and activating various signal transduction cascades, such as mitogen-activated protein kinase (MAPK), protein kinase C, and phosphatidylinositol 3-kinase (PI3K). This type of interaction could explain the so-called "non-genomic actions" of estrogen [53] (Figure 3). ER α , the major isoform in breast tissue, plays an essential role in normal mammary gland development and function as well as in breast cancer initiation and growth [56]. ER α is bound by the natural hormone, E2, which has a pleiotropic effect and is responsible for the proliferation and survival of breast epithelial cells. Therefore, in the case of ER-positive breast cancer, ER α is a good prognostic marker and a prime target for therapy. The antagonist effect of glyceollins on ER activity was first discovered in 2001 in both HEK 293 and MCF-7 cells [27]. More recent studies confirmed this antiproliferative effect of glyceollins in E2-dependent proliferative cell models [25,28]. Not only were their molecular modes of action better described, but also the glyceollin isomers were studied in isolation.

Some phytoestrogens, such as coumestrol, daidzein, and genistein, are partial agonists of ERs. They have ER-agonist activity in low-estrogen conditions, but ER-antagonist activity in high-estrogen conditions. Unlike some phytoestrogens, the mixture of glyceollins shows only antagonist effects on ERs in low-estrogen conditions [27,28]. These compounds have a greater affinity for ER α than ER β [27], which shows their potential to treat breast cancer, where ER α is the major isoform.

GI is the most potent antiestrogenic isomer in the glyceollin mixture [28,57]. GI has the strongest capacity to bind to ER α due to its highest binding affinity for ER α . Examinations of the interaction between GI, GII, and GIII and the ER α ligand binding cavity in docking studies demonstrated that GI interacts with ER α in a way that is similar to tamoxifen, whereas GII and GIII can only bind to ER α in a completely different way [28]. Among glyceollins, GI is also the strongest inhibitor of ER transcriptional activity and colony formation in MCF-7 and BG-1 cells [28]. Its effects are different from that of its estrogenic precursor, glycinol [58]. Among GI enantiomers, (+)-GI (synthetic enantiomer) slightly increased ERE activity, while (-)-GI (natural enantiomer) decreased the activity of both ER subtypes stimulated by E2, demonstrating potent anti-estrogenic properties [57].

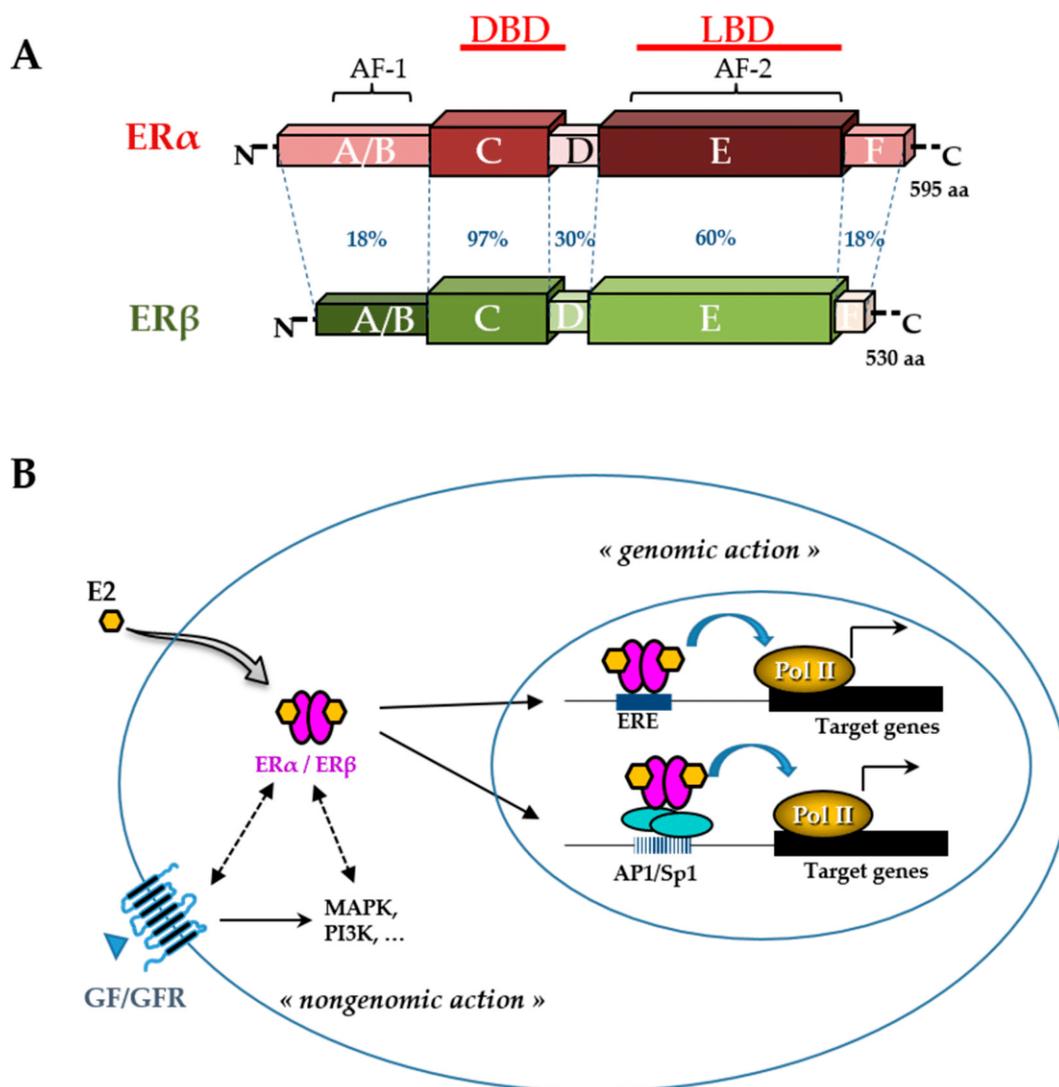


Figure 3. Estrogen receptor (ER) structure and action. The schematic structures of the two human ER α and ER β and the percentage of homology between the different domains (annotated by the letters A to F) are indicated (A). Domains involved in DNA binding (DBD), ligand binding (LBD), ligand-independent transactivation function 1 (AF-1), and ligand-dependent transactivation function 2 (AF-2) are shown. The number of amino acids for each receptor is also indicated on the right side. Estradiol (E2) mediates numerous phenotypic effects in cells by binding to and activating ERs (B). E2 enters the cell through the lipid membranes and binds ER, which can be present in the cytoplasm and the nucleus. The activated ER forms a dimer to tightly fix chromatin directly at the estrogen-responsive element (ERE) sites or indirectly at activator protein 1 (AP1) or specificity protein 1 (Sp1) sites. ER is then able to remodel chromatin by recruiting cofactors and activating RNA polymerase II (Pol II), at target genes (genomic action). Besides, ERs can use rapid non-genomic action through the interaction with intracellular kinases (mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K), ...) and the growth factor (GF) receptor (GFR) pathways.

As a transcription factor, ER α either directly or indirectly regulates the expression of many genes that are involved in cell growth and proliferation. Glyceollins inhibited the expression of the E2 target genes, PgR and CXCL12 (SDF-1), in MCF-7 cells [25,28]. A transcriptomic assay determined that the antiproliferative effect of glyceollins in ER-positive breast cancer cells was achieved through the ER and forkhead box M1 (FOXM1) factor pathway [25]. Glyceollins downregulated FOXM1, which is a well-known key regulator of the cell cycle, involved in G1/S and G2/M transitions (Figure 4).

Glyceollins can suppress ER transcriptional activity independently of the ligand binding domain and ER α phosphorylation. Glyceollins suppress the phosphorylation of proteins known to crosstalk with ER signaling, specifically protein S6 kinase, 70 kDa (p70S6K) [59]. As one of the best characterized downstream targets of mTOR, p70S6K is an important regulator of cell size, protein translation, and cell proliferation [60].

The antiestrogenic effect of glyceollins was also demonstrated *in vivo*. Indeed, mice xenografted with MCF-7 breast cancer cells and injected with a mixture of glyceollins showed a decrease in tumor growth [26,28]. Synthetic GI and GII decreased E2-dependent proliferation of mammary glands in ovariectomized mice [25]. In female monkeys, a glyceollin-enriched diet inhibited breast proliferation stimulated by E2 [40]. The expression of two ER-target genes in the breast epithelium, TFF1 and PgR, was markedly lower in the group with a glyceollin diet compared to that in the group treated with E2 alone.

The effect of glyceollins in other E2-dependent cell types or tissues was also investigated. GI inhibited colony formation of ovarian cancer BG-1 cells [28]. With their antiestrogenic action, glyceollins can inhibit the growth of human ovarian cancer xenografts [26]. After 20 days of the experiment, a treatment with a glyceollin mixture combined with E2 showed a reduction in BG-1 ovarian tumor volume (73.1%) when compared to E2 alone. These tumor-inhibiting effects corresponded to significantly lower E2-induced PgR expression in the tumors. While the use of tamoxifen or certain phytoestrogens in postmenopausal women with breast cancer is a concern because of the side effects of their estrogenic action on other tissues, such as the uterus [61], glyceollins did not show any estrogenic effects on the uterine morphology and partially antagonized the uterotrophic effects of estrogen in nude mice after 20 days of treatment [26]. However, in our recent study, a brief treatment via the injection of GI and GII alone for 3 days showed that these compounds had no antagonistic effect on the trophic action of E2 in mouse uteri [25]. The ability of glyceollins to function as an estrogen antagonist in the uteri of mice after a long-term treatment is a distinct advantage when compared with that of other compounds.

Furthermore, glyceollins exerted growth inhibitory effects on the human androgen-responsive prostate cancer cell line, LNCaP, by leading to G1/S arrest. Interestingly, this effect of glyceollins appeared to be mediated through the modulation of an estrogen-, but not androgen-mediated pathway [62].

4.2. Estrogen-Independent Effect

In addition to their ER-dependent antitumor effect, glyceollins could also suppress mammary tumorigenesis through ER-independent pathways. For instance, *in vitro*, glyceollins decreased the proliferation of the ER-negative breast cell line, MCF10A [25], and *in vivo* decreased MDA-MB-231 and MDA-MB-468 breast tumor volume in xenografted mice [63].

One potential mechanism for metastatic spread is the epithelial to mesenchymal transition (EMT) [64]. Characteristic of these EMT cells is a loss of E-cadherin expression and high expression of the transcription factor, ZEB1 [65]. The ZEB1 transcription factor, known as an inducer of EMT in cancer metastasis, acts through the transcriptional repression of E-cadherin. When letrozole-resistant breast cancer cells (LTLT-Ca) were treated with GI, they exhibited morphological characteristics similar to an epithelial phenotype, and the GI treatment decreased proliferation by increasing E-cadherin and decreasing ZEB1 [31]. These results demonstrated that GI could reverse EMT. In another study in MDA-MB-231 cells, glyceollins significantly increased miRNAs involved in EMT (miR-22, miR-29b, miR-29c, miR-30d, miR-34a, and miR-195) and tumor suppressors (miR-181c and miR-181d). There was also a significant decrease in the expression of oncomiRs promoting tumorigenesis (miR-21 and miR-193a-5p), oncomiRs promoting metastasis, such as miR-185, miR-224, and miR-486-5p, which are involved in cell migration and invasion, and miR-542-5p, which is involved in the maintenance of a mesenchymal phenotype (Figure 4).

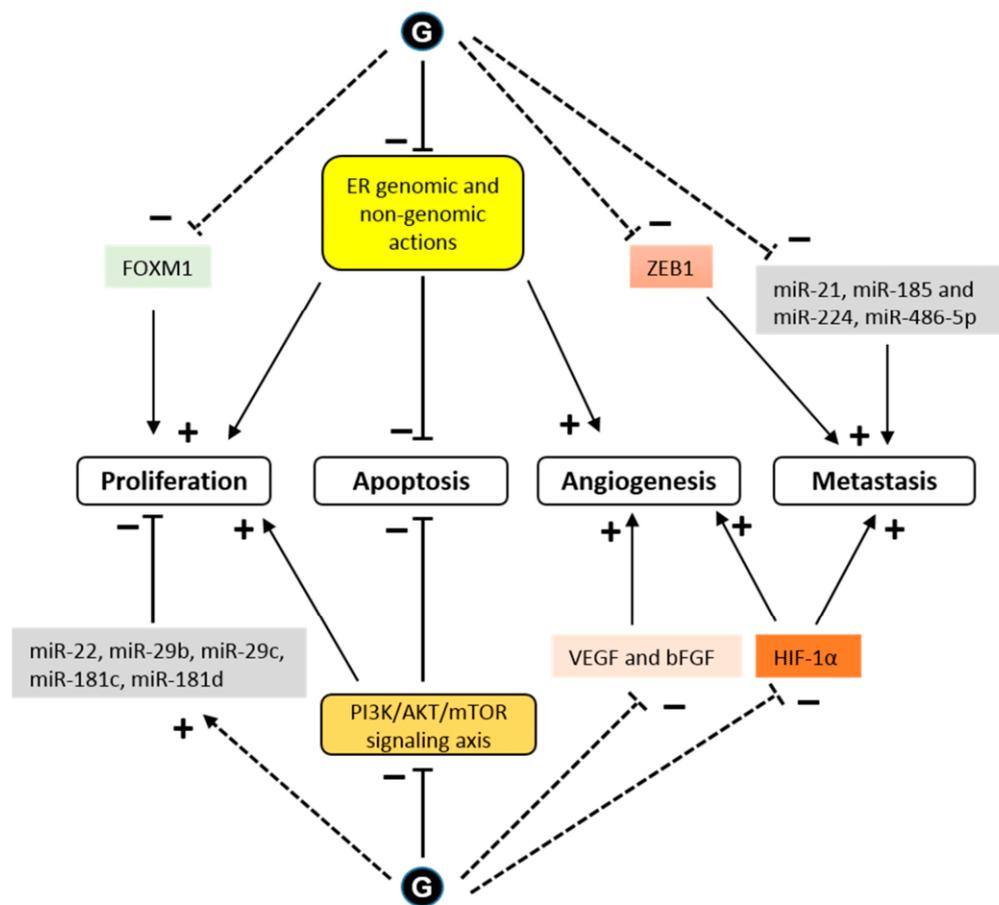


Figure 4. Signaling pathways modulated by glyceollins in the context of cancer cells. Glyceollins (G) have been shown to directly interact with the estrogen receptor (ER), exerting antagonistic effects on ER-dependent pathways. This anti-estrogenic effect of glyceollins prevents E2-dependent proliferation and angiogenesis. In addition, glyceollins induce cell apoptosis by direct ER genomic or nongenomic (membrane-initiated) effects. The expression of forkhead box M1 (FOXM1), a key regulator of the cell cycle, is downregulated by glyceollins. Cell growth and apoptosis can also be affected by glyceollins through ER-independent pathways. Glyceollins inhibit the activity of cytoplasmic kinases, such as the phosphatidylinositol 3-kinase/protein kinase B/mammalian target of rapamycin (PI3K/AKT/mTOR) signaling axis. Glyceollins repress the expression of growth factors, such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), and promote the expression of microRNAs (miRs) that act as tumor suppressors. Glyceollins also inhibit cell invasion and metastasis. These effects could be partially mediated by the inhibition of zinc finger E-box binding homeobox 1 (ZEB1) and hypoxia inducible factor 1 (HIF-1) expression as well as of microRNAs that enhance tumorigenesis (see text for references). Solid and dashed lines indicate direct and indirect effects, respectively. (+) indicates promoting effect and (-) indicates inhibiting effect.

The formation of microvessels is a critical step in the progression of cancer [66]. Multiple growth factors and cytokines existing in the tumor microenvironment contribute to angiogenic processes. Among them, VEGF and basic fibroblast growth factor (bFGF) are the major angiogenic factors induced by hypoxia. *In vitro*, glyceollins inhibited both VEGF- and bFGF-induced angiogenesis [29]. *In vivo*, glyceollins strongly blocked angiogenesis in zebrafish and chick embryos as well as in mice xenografted with lung cancer cells [29]. This effect of glyceollins could be useful not only in the case of cancers, but also for other angiogenesis diseases.

HIF-1 α is a transcription factor that is constitutively expressed. Under normoxic conditions, HIF-1 α is rapidly degraded by the proteins, prolyl hydroxylase domain (PHD) and factor inhibiting HIF (FIH-1) [67]. Under hypoxic conditions, HIF-1 α is stabilized and exerts its transcriptional activity.

The presence of active heat shock protein 90 (HSP90) is also necessary for the rapid accumulation and activation of HIF-1 α in both normoxic and hypoxic conditions [68]. There is an association between the stability of HIF-1 α and tumor growth [69]. Lee et al. showed that glyceollins potently inhibited HIF-1 α synthesis and decreased its stability by blocking the PI3K/AKT/mTOR pathway and HSP90 binding activity, respectively, under hypoxic conditions [30]. However, surprisingly, our recent study showed that glyceollins induced HIF-1 α under normoxic conditions [25]. HIF-1 α is controlled by numerous stimuli, including reactive oxygen species (ROS) [70], whose production is activated by glyceollins at high concentrations [71]. The effect of glyceollins on HIF-1 α could be due to its role in increasing the expression of REDD1, an mTORC1 inhibitor [25]. The inhibition of mTORC1 could be a factor involved in the antiproliferative effects of glyceollins through altering the PI3K/AKT/mTOR pathway (Figure 4).

The anticancer effect of glyceollins may be linked to their antioxidant effect, as discussed hereafter. Pretreatment or cotreatment with glyceollins protected mice from 7,12-dimethylbenz(a)anthracene-induced mammary tumorigenesis by reducing tumor formation and increasing the survival rate [72]. This protective effect was mainly associated with their potential to induce phase 2/antioxidant enzymes that play an essential role in enhancing the hydrophilicity of exogenous carcinogens to excrete them into bile or urine. However, at high concentration, glyceollins stimulated the production of ROS, which are possibly responsible for the apoptotic activity of the compounds [71]. Treatment with glyceollins at a high dose decreased cell viability and the mitochondrial membrane potential and increased DNA fragmentation. With their apoptotic potential, glyceollins could be exploited as antitumorigenic agents.

5. Other Effects in Noncancer Diseases

5.1. Osteoporosis

Osteoporosis is the most common bone disease in humans, representing a major public health problem, especially in menopause aged women [73]. Osteoporosis is defined pathologically by an imbalance between bone resorption and bone regeneration [74]. Therapeutic compounds for osteoporosis are divided into two main groups: Anti-resorptive drugs and anabolic drugs [74,75]. Anti-resorptive drugs reduce bone loss while anabolic drugs increase bone formation. E2 reduces osteoporosis through both anti-resorptive and anabolic mechanisms, but its use is disputed due to concerns about the risk of developing breast and endometrial cancers after this therapy [76,77]. Phytoestrogens can lead to promising alternative therapeutic strategies because they have shown the capacity to inhibit the bone resorption activity of osteoclasts, to stimulate osteogenic differentiation, and to increase the maturation of bone marrow-derived mesenchymal stem cells and osteoblasts [78]. A recent study from Bateman et al. examined the effect of glyceollins on the osteogenesis of adipose-derived stromal cells and bone marrow stromal cells [76] (Table 2). The treatment of these cell models with GI and GII showed increased calcium deposition relative to that of vehicle-treated cells. Interestingly, GII was more efficient in osteogenesis than E2 and GI. This effect of GII was partially inhibited by the antiestrogen, fulvestrant, indicating that the osteoinductive effect of GII is partly mediated by ER pathways. An analysis of gene expression demonstrated that GII increased the expression of genes involved in osteogenic differentiation in a similar manner to E2. Thereby, glyceollins are promising for development to become an anabolic drug in osteoporosis treatment.

Table 2. Glyceollins effects in noncancer diseases.

Diseases or Functions	Cell Line/Animal Model	Treatment	Major Effects	References
Osteoporosis	Adipose-derived stromal cells and bone marrow stromal cell line	GI ¹ and GIIT ²	- GI and GIIT: Increase calcium deposition - GI: Stronger than E2 and GI, increase the expression of genes involved in osteogenic differentiation in a similar manner to E2	Bateman et al. 2017 [76]
	Endocrine NCI-H716 cell line	Glyceollin mixture ³	- Enhance GLP-1 ⁴ secretion to increase insulinotropic actions	Park et al. 2010 [79]
Glucose and lipid metabolism	3T3-L1 adipocyte cell line	Glyceollin mixture	- Increase both insulin-stimulated and basal glucose uptake - Increase glucose transporter GLUT4 ⁵ level - Decrease triacylglycerol accumulation	Boué et al. 2012 [41], Park et al. 2010 [79]
	Insulina Min6 cell line	Glyceollin mixture	- Decrease apoptosis - Enhance insulinotropic actions	Park et al. 2010 [79]
	Diabetic male C57BL/6j mouse	Fermented soybeans containing glyceollins	- Decrease blood glucose level - Increase hepatic glycogen accumulation - Decrease triglyceride storage	Park et al. 2012 [42]
	Prediabetic male ZDSD/Peo rat	Glyceollin mixture	- Decrease blood glucose level	Boué et al. 2012 [41]
	Male golden Syrian hamster	Diet containing glyceollins	- Reduce plasma VLDL ⁶ , hepatic cholesterol esters, total lipid content - Alter expression of the genes related to cholesterol in liver	Huang et al. 2013 [80]
Female monkey (Macaca fascicularis)	Diet containing glyceollins	- Decrease serum total cholesterol, specifically LDL ⁷ and VLDL, increase serum triglycerides - Upregulate genes expression of PPAR ^{8,γ} , adiponectin, leptin, lipin 1, lipoprotein lipase and triglyceride	Wood et al. 2012 [81]	
Oxidative stress	Tests in vitro	Glyceollin mixture	- Have ferric-reducing antioxidant power, radical scavenging activities	Kim et al. 2010 [82]
	Hepa1c17/ BPRc1 cell line	Glyceollin mixture	- Inhibit H ₂ O ₂ -induced ROS ⁹ production - Induce Nrf2-mediated phase 2 detoxifying enzymes - Activate Nrf2-signaling pathway under oxidative stress	Kim et al. 2010 [82], Kim et al. 2011 [83], Jung et al. 2013 [84]
	Female C57BL/6j mouse	Glyceollin mixture	- Increase NAD(P)H:quinone oxidoreductase 1 activity in kidney, liver, and large intestine	Kim et al. 2015 [72]
	Rat	Glyceollin mixture	- Inhibit lipid peroxidation in liver, kidney, and brain tissue extracts	Kim et al. 2010 [82]

Table 2. Contd.

Diseases or Functions	Cell Line/Animal Model	Treatment	Major Effects	References
Central nervous system	Breast cancer MCF-7 cell line	Glyceollins mixture	- Increase nerve growth factor receptor gene expression	Zimmermann et al. 2010 [28]
	Glutamate-sensitive murine hippocampal HT22 cell line	Glyceollin mixture	- Attenuate glutamate-induced neurotoxicity - Suppress glutamate-induced intracellular ROS - Act through Nrf2 signaling pathway, activate hem oxygenase-1 enzyme	Seo et al. 2018 [85]
Primary cortical neurons from wild-type and Nrf2 knockout male C57BL/6j mouse	Primary cortical neurons from wild-type and Nrf2 knockout male C57BL/6j mouse	Glyceollin mixture	- Suppress glutamate-induced cytotoxicity in primary cortical neurons of wild-type mice, but not cells from Nrf2 knockout.	Seo et al. 2018 [85]
	Wild-type and Nrf2 knockout male C57BL/6j mouse	Glyceollin mixture	- Improve cognitive deficits caused by scopolamine in wild-type mice, but no effect in Nrf2 knockout mice - Inhibit acetylcholine esterase activity in neurons from the cortex, but not in cells from the hippocampus	Seo et al. 2018 [85]
Ovariectomized adult female CFW mouse	Glyceollin mixture	- Upregulate genes involved in neurogenesis, synaptic plasticity, tissue development and transcripts involved in signal transduction pathways, regulation of nerve impulse, cytoskeletal remodeling, and hormone signaling - Downregulate genes involved in neurodegeneration apoptosis and transcripts involved in developmental neurogenesis, synaptogenesis, and cell adhesion - Act through ER ¹⁰ -dependent or ER-independent mechanisms depending on the target genes	- Inhibit nitric oxide production and inflammatory mediators release through the NF- κ B pathway - Reduce HMGBl ¹¹ protein level induced by LPS ¹² - Decrease HMGBl-induced vascular barrier disruption - Decrease HMGBl-mediated pro-inflammatory and inflammatory response	Banji et al. 2015 [86], Banji et al. 2018 [87]
Inflammation	Murine macrophage RAW264.7 cell line	Glyceollin mixture	- Reduce mouse ear swelling caused by 12-O-tetradecanoylphorbol-13-acetate	Kim et al. 2011 [88], Yoon et al. 2012 [89]
	Human umbilical vein endothelial cell line	Glyceollin mixture	- Reduce HMGBl ¹¹ protein level induced by LPS ¹² - Decrease HMGBl-induced vascular barrier disruption - Decrease HMGBl-mediated pro-inflammatory and inflammatory response	Lee et al. 2014 [90]
ICR mouse	ICR mouse	Glyceollin mixture	- Reduce mouse ear swelling caused by 12-O-tetradecanoylphorbol-13-acetate	Kim et al. 2011 [88]
	Male C57BL/6 mouse that underwent cecal ligation and puncture	Glyceollin mixture	- Reduce serum HMGBl level, decrease HMGBl-induced vascular barrier disruption - Reduce HMGBl-induced mortality	Lee et al. 2014 [90]
Cardiovascular diseases	Human aortic smooth muscle cell line	Glyceollin mixture	- Inhibit the cell proliferation and migration induced by PDGF-BB ¹³	Kim et al. 2012 [91]
	Aortic rings from male Sprague-Dawley rat	GI	- Reduce vascular contraction partly through the inhibition of the RhoA/Rho-kinase signaling pathway	Song et al. 2010 [92]
Melanogenesis	B16/B16F10 cell lines	Glyceollin mixture	- Inhibit melanin synthesis by decreasing melanogenic enzymes and other factors	Lee et al. 2010 [93], Shin et al. 2013 [94]
	Standard AB strain zebrafish (<i>Danio rerio</i>) embryos	Glyceollin mixture	- Decrease the pigmentation of the embryos, melanin synthesis and tyrosinase activity - Decrease Sox10, a pigment cell-specific gene	Shin et al. 2013 [94]

¹ Glyceollin I, ² Glyceollin II, ³ Glyceollin mixture contains glyceollin I, glyceollin II, and glyceollin III, ⁴ glucagon-like peptide 1, ⁵ glucose transporter 4, ⁶ very low-density lipoprotein, ⁷ low-density lipoprotein, ⁸ peroxisome proliferator-activated receptor, ⁹ reactive oxygen species, ¹⁰ estrogen receptor, ¹¹ high mobility group box 1, ¹² lipopolysaccharide, ¹³ platelet-derived growth factor BB.

5.2. Glucose and Lipid Metabolism

Glyceollins have shown potential benefits in glucose and lipid metabolism (Table 2). In mice, the intake of a diet with 0.41 mg/day of glyceollins for 8 weeks decreased fasting serum glucose, increased fasting serum insulin, and decreased serum nonesterified fatty acids in streptozotocin-induced diabetic mice [42]. These effects of a decrease in blood glucose were also observed in prediabetic rats [41]. A diet with glyceollins reduced insulin resistance by as much as did rosiglitazone, a commercial insulin sensitizer [42]. In more detail, in the liver, the glyceollin diet improved insulin signaling and glucose metabolism by increasing the phosphorylation of protein kinase B (Akt) and AMP-kinase and by decreasing the phosphorylation of acetyl-CoA carboxylase and the expression of phosphoenolpyruvate carboxykinase [42]. In adipocytes, glyceollins improved insulin-stimulated glucose uptake and decreased triacylglycerol accumulation [79]. The stimulation of glucose uptake was due to an increase in glucose transporter 4 (GLUT4) transporters [41]. In the pancreas, the glyceollin diet partly increased the insulin secretion capacity by increasing the β -cell area and mass in islets of Langerhans [42]. In insulinoma cells, glyceollins increased the insulin level and alleviated the palmitate-induced impairment of β cell function and apoptosis via attenuation of endoplasmic reticulum stress [79]. Glyceollins decreased the expression of genes involved in fatty acid synthesis that are normally decreased by estrogen.

In the golden Syrian hamster, which have similar lipid profiles and a similar level of susceptibility to dietary cholesterol as humans, glyceollin supplementation significantly decreased plasma very low-density lipoprotein (VLDL), hepatic cholesterol esters, and the total lipid content. Moreover, the glyceollin diet also altered the expression of genes related to cholesterol metabolism in the liver [80].

In female surgically postmenopausal cynomolgus macaques, diet containing glyceollins for 3 weeks resulted in lower serum total cholesterol level, specifically low-density lipoprotein (LDL) and VLDL, and increased serum triglycerides level [81]. For gene expression in mammary biopsies, the glyceollin diet affected genes involved in lipid and carbohydrate metabolism, notably it upregulated peroxisome proliferator-activated receptor (PPAR)- γ , adiponectin, leptin, lipin 1, and lipoprotein lipase [81].

Together, these results supported that glyceollins have beneficial effects in glucose and lipid metabolism. A diet containing glyceollins may help to improve metabolic diseases.

5.3. Antioxidant Effect

The oxidation of nutrients for a biological energy supply is essential to life for all aerobic organisms [95]. However, cells that benefit from oxidative respiration are also burdened by ROS [95]. However, healthy cells balance the formation and elimination of ROS [95]. When the concentration of free radicals exceeds a critical level and the ROS-homeostasis is disturbed, oxidative stress occurs [95]. Oxidative stress plays a critical role in many diseases, such as diabetes, cardiovascular and neurodegenerative diseases, chronic inflammatory diseases, and cancer [96]. Several compounds from natural sources, such as quinoa and amaranth grains [97], nut [98], cranberry [99], and soybeans [100], benefit health through their antioxidants activities. As previously discussed in Section 4.2 about the antitumor estrogen-independent effects, the antioxidant activity of glyceollins has been demonstrated in several studies (Table 2). Glyceollins showed strong reducing power, inhibited lipid peroxidation via their large capacity to scavenge radicals, and suppressed H₂O₂-induced ROS production in hepa1c1c7 cells [82]. Moreover, glyceollins have been reported to induce phase 2 detoxifying enzymes [83,84]. Experimental and docking analyses suggested that this action occurred through promotion of the nuclear translocation of the nuclear factor (erythroid-derived 2)-like 2 (Nrf2) by disrupting Keap1-Nrf2 complex formation [83,84]. In mice, glyceollin treatment increased NAD(P)H:quinone oxidoreductase 1 (NQO1) activity in the kidney, liver, and large intestine [72]. In the rat, glyceollins inhibited lipid peroxidation in the liver, kidney, and brain tissue extracts [82]. Since oxidative stress can be considered as one of the causes of many common human pathologies and of the aging process [96], glyceollins,

with their antioxidant effects *in vitro* and *in vivo*, could be further studied to become a preventive therapy for oxidative stress-caused diseases.

5.4. Effect on the Central Nervous System

Several studies showed the neuroprotective effects of glyceollins on the central nervous system (CNS) (Table 2). Recently, Seo et al. examined the neuroprotective effect of glyceollins through the activation of Nrf2 in two neuronal cell models [85]. The transcription factor, Nrf2, is the major regulator of the expression of different antioxidant enzymes, such as heme oxygenase 1 (HO-1). Using mouse primary cortical neurons treated with glutamate, they showed that glyceollins reduced glutamate-induced cytotoxicity in neurons expressing wild-type Nrf2, but not in Nrf2 knockout neurons. Likewise, glyceollins reduced the production of ROS, which are involved in neurodegeneration, by stimulating the expression of Nrf2 and HO-1 in a hippocampal neuronal HT22 cell line. In this model, glyceollins significantly reduced ROS production after the cells were exposed to glutamate. Moreover, in a mouse model of amnesia, glyceollins improved mnemonic and cognitive deficits caused by scopolamine in wild-type, but not in Nrf2 knockout mice. These results suggest that glyceollins' actions are likely mediated by the stimulation of the Nrf2 / HO-1 signaling pathway [85].

Estrogens regulate numerous functions in the brain, including reproduction, food intake, cognition, neuronal synaptic plasticity, and pain perception [87]. As phytoestrogens, glyceollins may have effects in brain functions through ERs. Bamji et al. investigated the effect of glyceollins in a female mouse brain using microarray experiment [86] and paired-end RNA sequencing (RNA-seq) [87]. They showed that the actions of glyceollins in the CNS could be mediated by ER-dependent and ER-independent mechanisms depending upon the target gene. The transcripts regulated by E2 and glyceollins alone or in combination annotated to similar pathway maps and network in both microarray and RNA-seq experiments. In microarray, they showed that glyceollins upregulated genes involved in neurogenesis, synaptic plasticity, and tissue development [86]. In particular, the glyceollins downregulated the expression of the gene encoding peptidylprolyl isomerase A (PPIA), which regulates several biological processes, including inflammation and apoptosis. PPIA stimulates neurodegeneration and the loss of synaptic connections by activating NF- κ B and metalloproteinase 9 [101]. In the RNA-seq experiment, glyceollins upregulated transcripts involved in signal transduction pathways, regulation of nerve impulse, cytoskeletal remodeling, and hormone signaling, and downregulated transcripts involved in developmental neurogenesis, synaptogenesis, and cell adhesion [87]. Although these results suggested that glyceollins may potentially have neuroprotective effects by regulating the gene expression related to these pathways, the mechanism through which glyceollins affects gene expression in the brain is still unclear.

Using an *in vitro* cell model for neuronal differentiation [102], we tested the ability of glyceollins to enhance nerve growth factor (NGF)-induced neuritogenesis in the PC12 cell line (Figure 5). We have previously shown that PC12 cells stably transfected with ER α (PC12-ER) are able to develop a pseudoneuronal phenotype (neurite outgrowth) when treated with NGF [103–105]. As shown in Figure 5, our data clearly showed that GI and GII fully contributed to the neuritogenesis effect of NGF in PC12-ER (Figure 5), but not in PC12 control cells (PC12-Cont) that do not express ER α (Figure 5 –cartridge). This indicates that the effect of glyceollins depends on the presence of ER. It is interesting to note that Zimmermann et al. [28] previously reported that glyceollins strongly increased the expression of the nerve growth factor receptor (NGFR) gene, a member of the tumor necrosis factor receptor superfamily. This may explain the effect of glyceollins on neurite outgrowth in the PC12 model. Thus, although many more studies are needed to understand the effects of glyceollins on the CNS, these compounds might be used in the development of therapeutic treatments for CNS diseases.

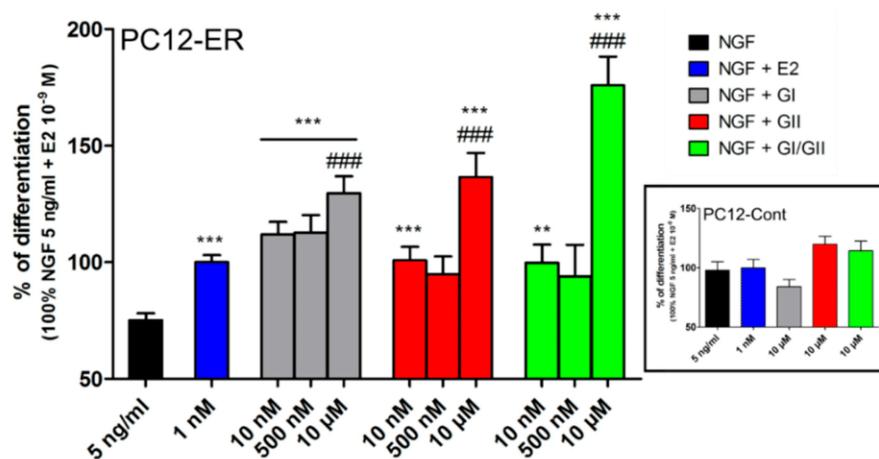


Figure 5. Glyceollins enhance the NGF effect on PC12-ER differentiation. PC12-ER transfected with ER α and PC12-Cont transfected with empty plasmid (cartridge) cells were treated for 72 h with 5 ng of nerve growth factor (NGF) associated with solvent (black), E2 10⁻⁹ M (blue), various concentrations of glyceollin I (GI, gray) or glyceollin II (GII, red), or a mixture (1:1) of both GI and GII (green). PC12 cells were then photographed to determine the percentage of cells bearing neurites. The results are expressed as the percentage of cells bearing neurites in the presence of NGF + E2. The results are the mean of at least three independent experiments \pm SEM. ** *p*-value < 0.01 and *** *p*-value < 0.001, with one-way ANOVA and Bonferroni's post hoc test used to compare the control cells (NGF + solvent) with the other treatments. ### *p*-value < 0.001, with one-way ANOVA and Bonferroni's post hoc test used to compare the NGF + E2-treated cells with the other treatments.

5.5. Anti-Inflammatory Activity and Cardiovascular Disease

Inflammation is a complex biological protective response against bodily injury [106]. However, continuous and chronic inflammation is linked to many chronic diseases, including cancer, cardiovascular diseases, obesity, and type 2 diabetes [107,108]. Glyceollins decreased inflammatory reactions caused by LPS in a murine macrophage cell line (RAW264.7) by inhibiting nitric oxide (NO) production and inflammatory mediators release [88,89]. Glyceollins inhibited IL-6 release and reduced the expression of iNOS and COX2 [88]. Moreover, glyceollins inhibited the expression of other pro-inflammatory cytokines, such as IL-1 β , IL-18, and TNF- α , and upregulated the expression of anti-inflammatory cytokines, including IL-10, in the RAW264.7 cell line [89]. This action was found to occur through the NF- κ B pathway. In vivo, glyceollins reduced mouse ear swelling caused by TPA [88].

Moreover, glyceollins have anti-septic effects via inhibition of the high mobility group box 1 (HMGB1) signaling pathway [90]. The biological activity of HMGB1 depends on its location, context, and post-translational modifications. However, a high level of circulating HMGB1 has been found in various diseases, particularly severe inflammatory diseases [109]. In the human umbilical vein endothelial cell line, glyceollins reduced HMGB1 protein levels induced by LPS. Consequently, glyceollins decreased the HMGB1-mediated pro-inflammatory and inflammatory response as well as vascular barrier disruption [90]. In mice, glyceollin treatment reduced serum HMGB1 level, HMGB1-induced vascular barrier disruption, and mortality [90]. Hence, glyceollins could be potential therapeutic agents for severe vascular inflammatory diseases, such as sepsis and septic shock.

The inflammatory process is often implied in cardiovascular diseases [107]. Glyceollins have shown their beneficial effects in these diseases not only through their anti-inflammatory effect, but also through other pathways (Table 2). GI reduced vascular contraction in an endothelium-independent manner partly through the inhibition of the RhoA/Rho-kinase signaling pathway in rat aortic rings [92]. Since the RhoA/Rho-kinase pathway plays a crucial role in cardiovascular diseases, such as vasospasm, arteriosclerosis, ischemia/reperfusion injury, hypertension, and heart failure [110], GI, with its vasorelaxant activity, can be studied as a cardiovascular therapeutic agent.

Platelet-derived growth factor (PDGF)-BB can induce the abnormal proliferation and migration of aortic smooth muscle cells (ASMC), which play an important role in the formation and development of atherosclerosis [111]. Kim et al. showed that glyceollins could inhibit the proliferation and migration of human ASMC induced by PDGF-BB [91]. Glyceollin treatment diminished the increased cell number and DNA synthesis caused by PDGF. This effect came from the capacity of glyceollins to block the PDGF-induced G₀/G₁ to S phase transition of the cell cycle, downregulating the expression of cyclin-dependent kinase (CDK)2 and cyclin D, and upregulating the expression of CDK inhibitors, such as p27^{kip1} and p53. Moreover, glyceollins inhibited the PDGF-induced dissociation of actin filaments and cell migration.

Furthermore, the antioxidant and cholesterol-lowering effects of glyceollins can also have an effect in cardiovascular diseases.

Briefly, glyceollins have anti-inflammatory activities through many different pathways. Therefore, they could be potential therapeutic agents for several inflammatory diseases, such as cardiovascular diseases, obesity, and type 2 diabetes.

5.6. Anti-Melanogenesis Activity

Melanin constitutes a group of natural dark pigments produced by a group of specialized cells known as melanocytes. In human skin, melanin plays an essential role in protecting the skin from DNA damage caused by ultraviolet radiation [112]. However, the abnormal accumulation of melanin causes dermatological problems, such as freckles, solar lentigo, melisma, postinflammatory melanoderma, and cancer [113]. In B16 melanoma cells, glyceollins decreased melanin synthesis by inhibiting melanogenic enzymes, such as tyrosinase and tyrosinase-related protein (TRP)-1 [93]. Additionally, glyceollins diminished intracellular cyclic AMP (cAMP) levels stimulated by α -melanocyte-stimulating hormone [93]. Melanogenesis is regulated by numerous factors, among which the stem cell factor (SCF) and the microphthalmia-associated transcription factor (MITF) play an important role. SCF plays a critical role in regulating the life cycle of human melanocytes, and MITF controls the expression of the melanogenic enzymes, tyrosinase, TRP-1, and TRP-2 [114]. In B16F10 melanoma cells, glyceollins attenuated melanin synthesis, tyrosinase activity, and c-kit and Akt phosphorylation induced by SCF [94]. Moreover, glyceollins significantly decreased the expression and activity of MITF and cAMP induced by SCF [94].

The anti-melanogenesis activity of glyceollins was also confirmed in zebrafish embryos [94]. Glyceollin treatment decreased the pigmentation of the embryos, melanin synthesis, and tyrosinase activity of the embryos. This treatment also dramatically decreased the expression of *Sox10*, a pigment cell-specific gene, in the neural tubes of the trunk region of the embryos. These results revealed that glyceollins could be useful for the treatment of hyperpigmentation problems or skin-whitening agents for cosmetic use.

6. Conclusions

Epidemiological studies have emphasized that plant-based diets (vegetables, fruits, herbs, and whole grains) may be beneficial for the prevention of certain chronic diseases and may moderately decrease the risk of developing certain cancers [4,9,115]. Because of their various structures, polyphenols are involved in a large array of biological functions, including antioxidant, anti-inflammatory, antiproliferative, and antiangiogenesis effects [1]. Consequently, these properties are attracting increasing interest for the prevention and treatment of cancer and inflammatory, cardiovascular, and neurodegenerative diseases [116].

In this review, we summarized diverse aspects of glyceollins and their molecular actions, indicating that these natural compounds can be beneficial to human health by reducing the incidence of numerous diseases. At the molecular level, glyceollins have been shown to interact with ERs and act as weak estrogenic/anti-estrogenic compounds in a cell-dependent manner. It was suggested that glyceollins can behave like selective ER modulators (SERMs) that have partial agonist and antagonist

properties depending on the ER isoform or cellular context [117]. In our previous study [25], glyceollins acted essentially as antiestrogenic compounds via the direct inhibition of ER activities in the context of breast cells. Thus, the reason for this conflicting finding could be explained by the use of different cell models in these two studies [25,117]. Nevertheless, consistent with the possible SERM activity of these compounds, we showed here that GI and GII enhanced the differentiation of PC12 cells, as E2 did. Our genome-wide microarray performed with the MCF-7 breast cell line treated with synthetic GI and GII, alone or together with E2, identified the FOXM1/ER α and HIF-1 α /HIF-2 α signaling axes as the two major pathways affected by glyceollins [25]. This result could explain the antiproliferative effects of glyceollins on ER-positive and ER-negative cell lines. To date, two studies have used xenograft mouse models that were injected with breast or ovary carcinoma cells and treated with E2 and a mixture of glyceollins. The results showed that glyceollins inhibited E2-stimulation of tumor growth, suggesting that they might be helpful in breast and ovarian carcinoma therapies [26,28]. Furthermore, in mice and zebrafish models, glyceollins caused a reduction in microvessel density, suggesting a potential preventive effect for hypervascularization caused cancers [29]. Further studies on the antiproliferative effects of glyceollins in hormone-dependent and -independent cancers using nude mice xenografted with tumor cells and treated orally with glyceollins in combination with traditional therapeutic agents (e.g., Tamoxifen, Fulvestrant, Everolimus) would be beneficial. This characterization would be a first step in determining whether the combined action of glyceollins and traditional therapeutic agents is effective and whether it is possible to decrease the dose of chemotherapy. Furthermore, a precise assessment of the mechanisms by which these components prevent cancer cell growth as well as their potential actions on the epigenetic programming of ER-target genes should be investigated. In addition to their anticancer properties in prostate, breast, and ovarian carcinomas [26,62], glyceollins have also shown antioxidant and anti-inflammatory actions as well as inhibitory effects on tyrosine kinase activities, Akt phosphorylation, and ribosomal protein S6 kinase (p70S6K) phosphorylation [59]. Glyceollins could impact the metabolism of drugs and xenobiotics by regulating the expression of genes encoding cytochrome P450 phase I and other detoxifying enzymes ([25] and unpublished data). They have neuroprotective effects in vivo and in neuronal cell models. Additionally, as we have reported here, glyceollins enhance neuronal differentiation, resulting in neurite outgrowth in PC12 cells. This action depends on the presence of ER.

Although much work has been done, much work also remains to be done before we can consider using these compounds as preventive or therapeutic agents in human pathologies. Indeed, little is known about the bioavailability and pharmacokinetics of glyceollins in different tissues. Some polyphenols, such as resveratrol or apigenin, are poorly bioavailable and are rapidly metabolized in the intestine or liver [118,119]. More data are also needed in toxicological studies to determine the effective and nontoxic doses in vivo. In addition, to date, no clinical or epidemiological studies have been done about glyceollins treatment. So, clinical trials on the use of glyceollins as supplements are required to obtain accurate data on the efficacy of these compounds and on the correct dosage for biomedical applications in human health.

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2.4. Apigénine

2.4.1. Structure et synthèse

L'apigénine (ou apigénol) est une molécule phytochimique de la famille des flavones, une sous-classe des flavonoïdes. Elle est présente dans les plantes sous la forme d'aglycone et / ou ses C- et O-glycosides (détectés sous 6-C-et 8-C-glucoside, et 7-O-glucoside) , glucuronides, O-méthyléthers et acétylés dérivés [196] (Figure 15).

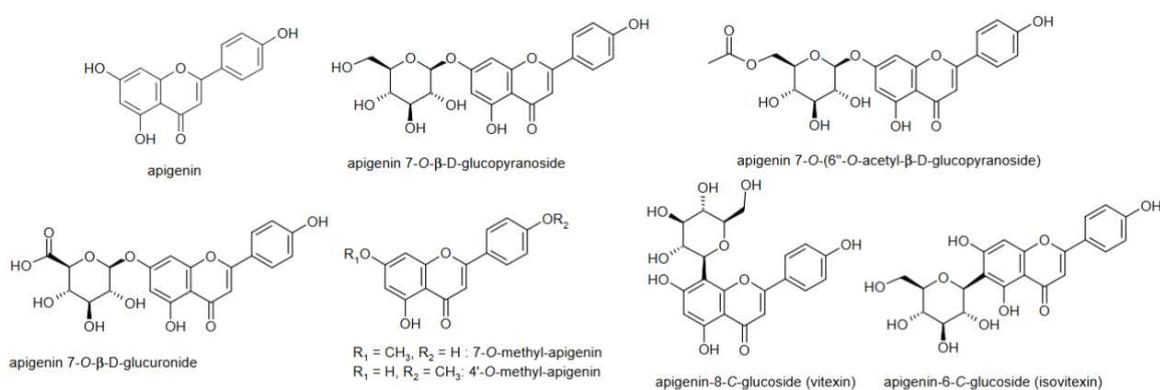


Figure 15. Structure de l'apigénine et de ses dérivés glycosidiques, glucuroconjugés, acétylés et esters méthyliques. Figure extraite et modifiée de l'article de Salehi *et al.* [196]

Biogénétiquement, l'apigénine est un produit de la voie des phénylpropanoïdes et peut être obtenue à partir de la phénylalanine et de la tyrosine, deux précurseurs dérivés du shikimate. À partir de la phénylalanine, l'acide cinnamique est formé par désamination non oxydante puis par oxydation en C-4, qui est ensuite converti en acide p-coumarique. À partir de la tyrosine, l'acide p-coumarique est formé directement par désamination. Après activation avec le CoA, le p-coumarate est condensé avec trois résidus malonyl-CoA puis aromatisé par la chalcone synthetase pour former la chalcone, qui est ensuite isomérisée par la chalcone isomérase pour former la naringénine. Enfin, une flavanone synthase oxyde la naringénine en apigénine [196] (Figure 16).

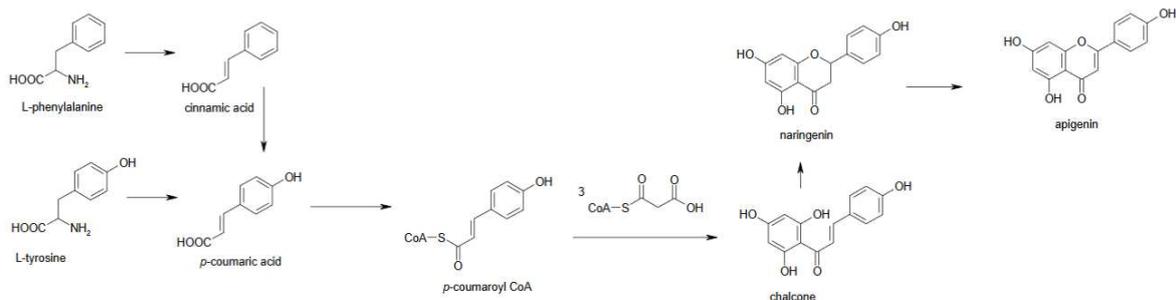


Figure 16. Voie biogénique de la biosynthèse de l'apigénine. Figure extraite et modifiée de l'article de Salehi *et al.* [196]

2.4.2. Source

Parmi les flavonoïdes, l'apigénine est l'une des molécules phénoliques les plus étudiées car elle est très répandue dans le règne végétal. Ce composé peut être trouvé en quantité significative dans les légumes, les fruits, les herbes, les céréales et les boissons à base de plantes. La concentration de l'apigénine en mg par 100 g de poids dans les différents aliments est présentée dans le tableau ci-dessous :

Tableau 5. Concentration de l'apigénine dans l'alimentation

Type d'aliment	Nom botanique	Nom commun	Apigénine (aglycones) ou glycosides	Concentration (mg/100 g de poids)
Thé et herbes sèches	<i>Chamaemelum nobile</i>	Fleurs de camomille romaine	Apigénine O-glycosides	2531
			Apigénine	395
	<i>Matricaria chamomilla</i>	Fleurs de camomille	Apigénine O-glycosides	5010–5320
	<i>Petroselinum crispum</i>	Persil	Apigénine/Apigénine O-glycosides	1200–1350
	<i>Tanacetum vulgare</i>	Feuille de tanaisie	Apigénine	165
	<i>Aspalathus linearis</i>	Rooibos	Apigénine C-glycosides	46–116
	<i>Trigonella foenum-graecum</i>	Graine de fenugrec	Apigénine	731
	<i>Mentha x piperita</i>	Menthe poivrée	Apigénine O-glycosides	<5,6–27
	<i>Origanum vulgare</i>	Origan	Apigénine	15,6–19,4
	<i>Perilla frutescens</i>	Pérille/Zisu	Apigénine O-glycosides	280–920
	<i>Rosmarinus officinalis</i>	Romarin	Apigénine	43,8
<i>Camellia sinensis</i>	Thé vert	Apigénine	47,8 –246,8	

		Thé noir	Apigénine	41,4–175,7
		Thé Oolong	Apigénine	198,6
Jus et vins	<i>Citrus bergamia</i>	Jus de bergamote	Apigénine C-glycosides	3,8–7,2
			Apigénine O-glycosides	6,1–7,7
	<i>Citrus deliciosa</i>	Jus de mandarine	Apigénine C-glycosides	2,3–2,7
	<i>Citrus medica</i>	Jus de citron	Apigénine C-glycosides	0,6–0,8
	<i>Citrus sinensis</i>	Jus d'orange	Apigénine C-glycosides	1,0–8,0
			Apigénine O-glycosides	0,2–0,7
<i>Vitis vinifera</i>	Vin rouge et blanc	Apigénine	<0,1–0,6	
Fruits, légumes, huile d'olive et miel	<i>Spinacia oleracea</i>	Épinard	Apigénine	<0,1
	<i>Petroselinum crispum</i>	Persil	Apigénine	<2,4–1484
	<i>Apium graveolens var, dulce</i>	Branches de céleri	Apigénine	1,3–10,8
		Cœurs de céleri	Apigénine	19,1
	<i>Apium graveolens var, secalinum</i>	Céleri chinois	Apigénine	24,0
	<i>Lactuca sativa</i>	Laitue	Apigénine	<0,7–2,7
	<i>Cynara scolymus</i>	Tête d'artichaut	Apigénine/Apigénine glycosides	3,9–18,9
	<i>Olea europaea</i>	Huile d'olive	Apigénine	<0,1–0,2
	<i>Fortunella crassifolia</i>	Kumquat	Apigénine glycosides	21,9
	Miel	Apigénine	<0,1–29,3	
Céréales et légumineuses	<i>Pisum sativum</i>	Pois cultivé	Apigénine C-glycosides	<0,3–4,6
	<i>Vicia faba</i>	Fèves	Apigénine C-glycosides	<0,3–0,5
			Apigénine O-néohespéridoside	<0,3–0,3
	<i>Digitaria exilis</i>	Fonio blanc	Apigénine	15,0
	<i>Sorghum bicolor</i>	Sorgo jaune-citron	Apigénine	0,1–28,7
		Sorgo rouge	Apigénine	<0,2–20,4
	<i>Triticum monococcum</i>	Graine de blé	Apigénine C-glycosides	2,1
	<i>Triticum urartu</i>	Graine de blé	Apigénine C-glycosides	3,8
<i>Triticum dicoccum</i>	Graine de blé	Apigénine C-glycosides	9,5	
<i>Triticum carthlicum</i>	Graine de blé	Apigénine C-glycosides	10,5	

	<i>Triticum polonicum</i>	Graine de blé	Apigénine C-glycosides	7,4
	<i>Triticum turgidum</i>	Graine de blé	Apigénine C-glycosides	12,7
	<i>Triticum durum</i>	Graine de blé	Apigénine C-glycosides	7,8
	<i>Triticum spelta</i>	Graine de blé	Apigénine C-glycosides	17,9
	<i>Triticum aestivum</i>	Graine de blé	Apigénine C-glycosides	8,0
	<i>Oryza sativa</i>	Riz noir	Apigénine C-glycosides	6,3
		Riz rouge	Apigénine C-glycosides	0,7
		Riz complet/ Riz brun	Apigénine C-glycosides	1,8
		Riz blanc	Apigénine C-glycosides	1,6
	<i>Fagopyrum esculentum</i>	Sarrasin	Apigénine C-glycosides	0,2-0,7

Données extraites de la revue de Hostetlet *et al.* [197]

2.4.3. Propriétés d'absorption, de distribution, de métabolisme et d'excrétion de l'apigénine

2.4.3.a. Absorption

L'absorption de l'apigénine après l'administration orale est considérée comme faible à cause de sa solubilité limitée dans les lipides et dans l'eau [198]. La forme aglycone est mieux absorbée que la forme glycoside correspondante [199]. Concernant la vitesse d'absorption de l'apigénine, des conclusions contradictoires ont été tirées. L'étude de Gradolatto *et al.* chez le rat suggère que l'apigénine a une absorption lente car après une administration orale unique d'apigénine radiomarquée, la radioactivité n'apparaît dans le sang que 24 h après l'administration [200]. Une autre étude a démontré au contraire que l'apigénine a un taux d'absorption rapide chez le rat, car la concentration plasmatique d'apigénine atteint le niveau maximal 3,9 heures après la prise orale sous forme d'extrait de *Chrysanthemum morifolium* [201]. L'apigénine peut être transportée par des mécanismes saturables passifs et actifs médiés par des transporteurs dans le

duodénum et le jéjunum, et par des mécanismes de transport passifs dans l'iléon et le côlon [202].

2.4.3.b. *Distribution*

Un fois absorbée, l'apigénine se distribue bien dans les tissus [199]. Dans l'étude de Gradolatto *et al.* mentionnée précédemment, après une seule administration orale d'apigénine radiomarquée, la demi-période d'élimination est élevée avec une valeur de 91,8 heures. Après 10 jours, 1,2% de la radioactivité est retrouvée dans le sang, 0,4% dans les reins, 9,4% dans l'intestin, 1,2% dans le foie et 24,8% dans le reste du corps [200]. Dans une autre étude, des souris ont reçu un régime contenant de l'apigénine pendant 7 jours et elle est détectée dans le plasma, le foie et la petite muqueuse intestinale avec une concentration stable à partir de 5 jours de traitement [203].

2.4.3.c. *Métabolisme*

L'apigénine absorbée peut passer par un métabolisme étendu de phase I et de phase II. Dans le foie du rat, le métabolisme de l'apigénine implique des enzymes de phase I en présence de NADPH (nicotinamide adénine dinucléotide phosphate), P450 (enzymes du cytochrome P450) ou FMO (monooxygénase contenant des flavines) [204]. La biotransformation de phase II de l'apigénine implique un cycle entérique et entéro-hépatique [201]. Les réactions de conjugaison glucuronidation et de sulfatation sont les voies métaboliques essentielles de la phase II de l'apigénine [204]. Chez le rat comme chez l'homme, il a été rapporté que l'apigénine produisait les principaux métabolites des conjugués glucuronidés et sulfatés [205]. Le principal métabolite hépatique de l'apigénine est la lutéoline [200].

2.4.3.d. *Excrétion*

Dans une expérience utilisant des rats, après une seule administration par voie orale d'apigénine radiomarquée, 51% de la radioactivité est retrouvée dans l'urine

et 12% dans les fèces après 10 jours [200]. Dans la même étude, il a été découvert que le sexe et l'âge des rats affectent l'élimination par voie urinaire des conjugués d'apigénine. Cette recherche a également conclu que l'apigénine a un métabolisme et une phase d'élimination lents. Ainsi, ce flavonoïde s'accumule probablement dans le corps.

2.4.3.e. Préparation pour augmenter la biodisponibilité de l'apigénine

Plusieurs études ont été réalisées pour trouver des préparations qui peuvent augmenter la biodisponibilité orale de l'apigénine. Une préparation d'une dispersion solide de nanopoudre de carbone développée par Ding *et al.* peut augmenter la solubilité de l'apigénine de 275% [198].

2.4.4. Résumé des effets de l'apigénine sur la santé humaine

L'apigénine est abondamment présente dans des herbes longtemps utilisées en médecine traditionnelle chinoise [206]. Les études *in vitro* et *in vivo* ont montré que l'apigénine avait de fortes activités anti-inflammatoires, antioxydantes, antibactériennes, antivirales et aussi réduisait la pression artérielle [206]. Par conséquent, cette molécule peut être un candidat prometteur pour prévenir et traiter les maladies chroniques comme les maladies cardiovasculaires, le diabète, l'obésité, les maladies neurodégénératives ou la dépression et l'insomnie [170,196].

De nombreux auteurs considèrent que l'apigénine pourrait constituer une option thérapeutique pour différents cancers [207–209] comme des cancers de la sphère uro-génitale (cancer du col utérin [210–213], des ovaires [214–218], cancer de la prostate [219–236]), les cancers du système digestifs (cancer de l'estomac [237–240], du colon [241–255], du pancréas [256–263], du foie [264–270]), les cancers de la bouche, de la tête et du cou [271–280], de la peau [281–287], du poumon [288–295], des os [296–298], du cerveau [299–301], la leucémie [302–308] ou encore le cancer du sein.

Dans le cadre de cette thèse, nous nous sommes intéressés aux effets de l'apigénine sur le CS. Ces aspects sont donc discutés plus en détails dans le sous-chapitre suivant.

2.4.5. Effets de l'apigénine sur le CS

2.4.5.a. CS récepteurs-positifs

De nombreuses études ont montré les effets anti-prolifératifs de l'apigénine dans les cellules MCF-7 ER-positives [309,310]. Zhou *et al.* ont montré que l'apigénine provoque l'apoptose et inhibe la survie clonogénique des cellules MCF-7 en supprimant l'expression de MUC-1 [309]. Son effet apoptotique a aussi été observé sur la lignée cancéreuse mammaire T47D [311].

Seo *et al.* ont montré que l'apigénine peut inhiber la prolifération des cellules cancéreuses du sein ER-positives en induisant l'apoptose par voie extrinsèque d'une part en induisant p53 et d'autre part en inhibant la signalisation STAT3 et NFκB [310]. La surexpression de HER2 dans ces cellules n'a pas conféré de résistance à l'apigénine. De plus, l'apigénine réduit la phosphorylation de HER2 [310]. Chez les souris greffées avec des cellules cancéreuses mammaires BT-474 HER2 /neu positives, l'apigénine inhibe la progression et le développement des tumeurs xéno-greffées en induisant l'apoptose, en inhibant la prolifération cellulaire et en réduisant l'expression de HER2 / neu [312].

2.4.5.b. CS triples négatifs

De nombreuses études ont démontré que l'apigénine diminuait de manière significative la prolifération et la migration des cellules de CS triple négatif (TNBC) *in vitro* et *in vivo* dans des modèles de xéno-greffe TNBC [180,311,313–315]. Chen *et al.* est la première équipe qui a montré les effets anti-prolifératifs et apoptotiques de l'apigénine sur les cellules MDA-MB-231 à la fois *in vitro* et *in vivo* en inhibant l'activité du protéasome [313].

Dans le TNBC, les cellules souches du CS sont très enrichies et liées à la résistance à la chimiothérapie, à la rechute tumorale et aux métastases [316,317]. Li *et al.* ont démontré que l'apigénine était capable d'inhiber les caractéristiques de cellules souches dans les cellules TNBC *in vitro* et *in vivo* en diminuant l'activité des cofacteurs transcriptionnels YAP / TAZ [314].

Les tumeurs mammaires à un stade avancé ont une concentration élevée de facteur de nécrose tumorale- α (TNF- α). Ce facteur provoque une libération prolongée de diverses chimiokines (par exemple CCL2 / CCL5) qui peuvent ensuite induire l'infiltration de masse de sous-populations de leucocytes qui viennent se loger dans la tumeur, déclenchant ainsi une perte de surveillance immunitaire tumorale et une croissance tumorale ultérieure rapide. *In vitro*, sur le modèle MDA-MB-231, Bauer *et al.* ont montré que l'apigénine supprime la libération du CCL2, facteur de stimulation des colonies de macrophages granulocytaires (GM-CSF), des interleukines IL-1 α et IL-6 par TNF α [318]. De plus, par analyse transcriptomique, cette équipe a montré que l'apigénine régulairement à la baisse 35 des 75 gènes régulés positivement par le TNF- α [319].

Outre le TNF- α , IL-6 est également considérée comme la pro-cytokine clé impliquée dans l'inflammation chronique dans le cancer, la progression tumorale et les métastases [320–322]. Les cancers du sein ER-négatifs produisent plus d'IL-6 que les cancers du sein ER-positifs, et une forte expression d'IL-6 est corrélée à des degrés et à des stades tumoraux plus avancés [323,324]. Lee *et al.* ont montré que le traitement par l'apigénine inhibait de manière significative la croissance des tumeurs de xéno-greffe MDA-MB-231 ainsi que l'expression des protéines de pSTAT3, pERK, IL-6, PI3K, pAkt et N-cadhérine [315]. Ces effets se sont révélés être médiés par l'inhibition de la voie de signalisation impliquant IL-6.

De nombreuses études ont montré des effets anti-migration et anti-invasion de l'apigénine sur le TNBC [314,325]. *In vitro*, l'apigénine diminue la migration et l'invasion des cellules MDA-MB-231 and MDA-MB-436 [314,325]. Ces effets sont dus à l'inhibition de la croissance et de la migration des métastases favorisées par le

« Hepatocyte growth factor » (HGF) par le blocage de la voie PI3K / Akt et la fonction de l'intégrine $\beta 4$ [325]. À un stade précoce de métastase, les cellules cancéreuses du sein envahissent le système vasculaire lymphatique et colonisent les ganglions lymphatiques. L'apigénine atténue cette intravasation de la barrière endothéliale [326].

L'activité aberrante des sirtuines (SIRTs) a de fortes implications dans la progression métastatique et oncogénique du TNBC [327–332]. Par l'utilisation de techniques de docking et des expériences *in vitro*, Sinha *et al.* ont montré que l'apigénine peut être un candidat pour devenir un inhibiteur des SIRTs [333].

2.4.5.c. CS résistants aux traitements

De nombreuses études ont montré que l'apigénine peut vaincre la résistance aux médicaments dans le cancer du sein. Cette résistance est causée par divers facteurs, notamment la surexpression de la glycoprotéine P (P-gp, MDR1). La P-gp protège normalement plusieurs organes des composés toxiques en les empêchant d'entrer dans le cytosol et en favorisant l'excrétion [334]. Cependant, chez les patients atteints de tumeurs, la P-gp excrète les agents chimiothérapeutiques des cellules, ce qui diminue leur efficacité [334]. En utilisant des cellules cancéreuses du sein résistantes à l'adriamycine (MCF-7 / ADR), Seo *et al.* ont montré que l'apigénine diminuait la croissance et la formation de colonies en induisant l'apoptose, en réduisant l'expression de P-gp et en inversant l'efflux de médicament des cellules MCF-7 / ADR [335]. Cet effet est lié à la suppression du transducteur de signal et de l'activateur de la voie de signalisation de la transcription 3 (STAT3).

La protéine de résistance au cancer du sein (BCRP) est un important transporteur d'efflux de la cassette de liaison à l'ATP. La surexpression de cette protéine dans les cellules sensibles aux médicaments confère une résistance au mitoxantrone, à la doxorubicine, à la daunorubine et au topotécan [336]. En testant 99 flavonoïdes, Fan *et al.* ont montré que 11 flavonoïdes, dont l'apigénine, entraînaient une inhibition significative de la BCRP, ce qui réduisait l'efflux médié

par la BCRP de doxorubicine et de témozolomide dans les cellules BCRP-MDCKII [337]. De plus, ces 11 flavonoïdes ont également augmenté l'aire sous la courbe (AUC) de la mitoxantrone chez le rat.

Le cisplatine est l'un des médicaments chimiothérapeutiques les plus couramment utilisés pour les tumeurs malignes, bien qu'une résistance à ce médicament puisse se produire. Liu *et al.* ont montré récemment qu'un co-traitement avec l'apigénine pouvait améliorer l'activité cytotoxique du cisplatine en induisant l'apoptose par la voie de signalisation de p53 [338].

Tableau 6. Synthèse des études de l'effet de l'apigénine sur le cancer du sein

Cancer du sein type / problème	Modèle expérimental	Dose de traitements	Résultats	Référence
Récepteurs positifs	MCF-7	25-100 μ M	Induction de la mort cellulaire apoptotique et perte de la survie clonogénique en supprimant l'expression de la mucine 1 (MUC-1)	[309]
	T47D	10; 20; 40 ou 80 μ M	Induction de l'apoptose et de l'autophagie	[311]
	BT-474 xélogreffe chez des souris nues athymiques femelles	50 mg/kg/jour pendant 21 jours	Inhibition de la progression et du développement des tumeurs xélogreffées en induisant l'apoptose et en réduisant l'expression de HER2/neu	[312]
	MCF-7 vecteur et HER2-surexprimée	10, 20 ou 40 μ M pendant 24h, 48h ou 72h	Inhibition de la prolifération cellulaire à la fois dans les cellules MCF-7 et MCF-7 HER2- surexprimées en induisant l'apoptose par voie extrinsèque en induisant p53 et en inhibant la signalisation STAT3 et NF κ B Réduction de la phosphorylation de HER2	[310]
Triple négatif (TNBC)	MDA-MB-231	25, 50, 75 ou 100 μ M pendant 24h	Inhibition de la prolifération cellulaire en induisant l'apoptose et en inhibant le protéasome	[313]
	MDA-MB-231 xélogreffées chez des souris nues athymiques femelles	25 ou 50 mg/kg/jour pendant 29 jours	Inhibition de la croissance tumorale en inhibant l'activité protéasomique et en induisant l'apoptose	[313]
	MDA-MB-231	40 μ M pendant 24h	Suppression de la libération de CCL2, facteur de stimulation des colonies de macrophages granulocytaires (GM-CSF), des interleukines IL-1 α et IL-6 par TNF α Diminution de l'expression de 35 des 75 gènes dont l'expression était augmentée par le TNF- α	[318,319]
	MDA-MB-231 MDA-MB-436	5, 10, 20 ou 40 μ M pendant 12h, 24h, 48h ou 72h	Inhibition de la prolifération cellulaire, de la formation de colonies et de la migration cellulaire; inhibition des propriétés des cellules souches en inhibant l'activité de YAP/TAZ	[314]
	MDA-MB-231 xélogreffées chez des souris nues femelles BALB/c	Prétraitement des cellules à 20 μ M pendant 48h avant	Inhibition du développement tumoral en inhibant les propriétés des cellules souches	[314]

		l'injection chez la souris		
	MDA-MB-231	10-50 μ M pendant 24h	Inhibition de la migration cellulaire et diminution de l'expression de Snail et de la N-cadhérine via l'inhibition de l'IL-6	[315]
	MDA-MB-231 xénotreffées chez des souris nudes BALB/c	25 ou 50 mg/kg/day pendant 14 days	Inhibition de la croissance et des métastases de la tumeur en réduisant l'expression de pSTAT3, pERK, IL-6 et pAkt	[315]
	MDA-MB-231	10, 20, 40 μ M pendant 24h	Inhibition de la motilité, de la diffusion, de la migration et de l'invasion cellulaire stimulée par le HGF en bloquant la voie PI3K/Akt et la fonction de l'intégrine β 4	[325]
	MDA-MB-231 injectées dans la veine de la queue sur des souris nudes femelles	Prétraitement des cellules à 40 μ M pendant 2h avant l'injection chez la souris	Suppression de métastases provoquées par le HGF des cellules MDA-MB-231 dans le poumon	[325]
	MDA-MB-231 injectées dans des embryons de poulet	Prétraitement des cellules avec 40 μ M pendant 2h avant l'ensemencement sur la membrane chorioallantoïde de poussin	Prévention des métastases spontanées provoquées par le HGF dans les embryons de poulet	[325]
	Docking MDA-MB-468	6,25 ; 12,5 ou 25 μ g/ml	Inhibition de l'expression et l'activité des sirtuines	[333]
	MDA-MB-231	10, 20 ou 40 μ M	Diminution de la croissance cellulaire et de l'intravasation à travers la barrière endothéliale	[326]
	MDA-MB-231	10; 20; 40 ou 80 μ M	Induction de l'apoptose et de l'autophagie	[311]
Résistance aux traitements	MCF-7, MCF-7 résistant à l'adriamycine (MCF-7/ADR)	0, 20, 40, 60, 80 ou 100 μ M	Réduction de la croissance cellulaire et de la formation de colonies dans les cellules MCF-7 parentales et MCF-7/ADR en induisant l'apoptose Diminution de l'expression de <i>multidrug resistance 1</i> (MDR1), <i>multidrug resistance-associated proteins</i> (MRPs) et P-gp in MCF-7/ADR cells. Réversion de l'efflux de médicament dans les cellules MCF-7/ADR	[335]

	MDCKII	2-70 μ M	Inhibition du BCRP et réduction de l'efflux médié par le BCRP de doxorubicine et de témozolomide	[337]
	Rat Sprague-Dawley mâle	Prétraitement de l'Api 25 mg/kg + Mitoxantrone	Augmentation de l'aire sous la courbe de la mitoxantrone à différents degrés chez le rat	[337]
	MCF-7	Prétraitement de l'Api à 30 μ M pendant 2h	Amélioration de l'activité cytotoxique du cisplatine	[338]

CONTEXTE ET OBJECTIFS DE LA THÈSE

Les travaux de thèse présentés dans ce manuscrit ont été produits pendant mes trois années passées à l'Irset (Institut de Recherche en Santé, Environnement et Travail) Inserm UMR1085 au sein de l'équipe Transcription, Environnement et Cancer (Trec). Ces travaux ont été réalisés sous la direction des Dr Farzad Pakdel, Dr François Ferrière et Dr Sylvain Lecomte.

Depuis de nombreuses années, les travaux de l'équipe se concentrent sur deux grands défis : étudier les mécanismes de la progression du CS pour aider à trouver de nouvelles cibles thérapeutiques et des marqueurs de pronostic ; et évaluer l'effet des facteurs environnementaux sur la santé. Trois axes de recherches sont développés dans l'équipe. Le premier axe concerne des mécanismes de la régulation génique et épigénétique au cours de la progression tumorale du CS en visant à comprendre la résistance hormonale. Le deuxième axe se focalise sur l'impact de l'exposition environnementale aux xénoœstrogènes sur la santé humaine. Le troisième axe s'applique à l'évaluation de l'impact des ondes électromagnétiques sur la santé humaine.

Mon activité scientifique de thèse s'inscrit dans la continuité des premier et deuxième axes de recherche de l'équipe. Les objectifs de ces travaux sont non seulement d'examiner l'effet potentiel des molécules naturelles sur la progression du CS mais aussi de mieux comprendre leurs mécanismes d'action en se concentrant sur les cibles thérapeutiques émergentes dans le CS avancé.

En effet, mon travail de thèse a été réalisé dans le cadre du projet mVolio financé par le Fond Unique Interministériel (FUI). Il était une collaboration entre différents partenaires académiques et industriels : l'équipe Trec (Transcription, Environnement et Cancer) du laboratoire Irset-Inserm UMR1085, le laboratoire Nutrinov (Groupe Triballat Noyal sur Vilaine), la société Agrène, le centre mondial d'Innovation du groupe Roullier et la société SATT Grand-Est et Welience (nouvellement nommée Sayens). Ce projet portait sur le développement de

composés bioactifs issus d'un procédé innovant de bioconversion de produits végétaux. Un des objectifs de ce projet était de mieux définir le mode d'action de ces composés bioactifs et de mieux comprendre leur potentiel œstrogénique/anti-œstrogénique ou SERM dans différents contextes cellulaires, notamment vis-à-vis du CS.

La première partie de ma thèse se concentre sur les glycéollins, qui étaient les molécules centrales du projet mVolio - FUI. Avant mon arrivée, l'équipe avait examiné des effets de ces molécules sur le CS ER-positif. Pour compléter cet aspect, mon travail avait pour but d'étudier les effets des glycéollins dans le CS triple-négatif par leurs actions sur la voie de signalisation d'AhR.

En parallèle de ces travaux sur les glycéollins, pendant ma première année de thèse, j'ai participé à l'étude des mécanismes d'action de l'apigénine et de la zéaralénone dans les cellules cancéreuses mammaires ER-positives. En observant les potentiels effets anti-cancéreux de l'apigénine dans cette étude, nous voulions mieux comprendre les actions de l'apigénine sur le CS ER-positif. Cela a donc conduit à la deuxième partie de ma thèse qui est consacrée à l'étude des effets de l'apigénine dans le CS ER-positif résistant à l'hormonothérapie.

Ces deux parties principales de ma thèse seront respectivement présentées en deux articles dans la partie résultats. En outre, vous trouverez, dans les annexes, un article sur les mécanismes d'action de l'apigénine et la zéaralénone et un article de vulgarisation concernant les SERMs, articles auxquels j'ai participé au cours de ma thèse.

Dans le contexte où les cas de CS triple-négatifs ou hormonorésistants sont encore incurables, nous espérons que ces travaux de thèse pourront contribuer au développement de nouveaux traitements pour ces types de CS.

RESULTATS

Partie 1: Caractérisation des glycéollins comme de nouveaux ligands d'AhR (aryl hydrocarbon receptor) et leur rôle dans la migration cellulaire

Dans cadre du projet mVolio – FUI, le laboratoire Nutrinov avec la SATT Grand-Est et Welience avaient développé une approche innovante de production de glycéollins. Mon équipe d'accueil avait intégré le volet santé humaine de ce projet afin de caractériser les propriétés biologiques de ces composés.

Dans le premier article publié par Sylvain Lecomte *et al.* [193], nous avons pu mettre en évidence l'effet anti-prolifératif des glycéollins à la fois sur la glande mammaire de souris et sur différentes lignées cancéreuses mammaires ER-positives et ER-négatives. Dans l'ensemble, cette étude a montré que les glycéollins exercent des effets anti-prolifératifs, médiés par ER, en agissant sur des gènes du cycle cellulaire. Les mécanismes d'action ont été trouvés à travers au moins deux voies de signalisations : FOXM1 et famille de HIF. Ces résultats ont renforcé le potentiel thérapeutique des glycéollins et ont confirmé l'intérêt de ces molécules dans la prévention ou dans le traitement du CS hormono-dépendant. Cependant, des études supplémentaires étaient nécessaires pour déterminer tous les mécanismes qu'impliquent les glycéollins. Notamment, dans l'analyse transcriptomique sur des cellules cancéreuses mammaires ER-positives, nous avons observé que l'expression de plusieurs gènes du métabolisme stéroïdien et xénobiotique était fortement modifiée par les glycéollins. Puisque AhR est un facteur clé dans la régulation de ces gènes, nous nous sommes demandé si les glycéollins étaient capables de se lier et d'activer AhR. De plus, des études récentes suggèrent fortement l'utilisation d'AhR comme cible thérapeutique dans le CS. Le premier objectif de la première partie de cette thèse est d'étudier l'effet des glycéollins sur la voie de signalisation d'AhR. Le deuxième objectif est d'examiner l'effet des glycéollins sur la migration cellulaire des cellules cancéreuses mammaires.

Pour étudier l'interaction entre le GI, le GII et AhR, une analyse informatique de docking, des analyses de luciférase, des immunofluorescences, des analyses de transcriptome et des PCR quantitatives ont été effectuées avec différentes lignées de cellules cancéreuses.

Les expériences de docking ont prédit que GI et GII peuvent entrer dans la poche de liaison d'AhR, mais leurs interactions avec les acides aminés du site de liaison diffèrent en partie de celles qui interagissent avec la 2,3,7,8-tétrachlorodibenzo-p-dioxine (TCDD). Deux modèles cellulaires standards, les cellules HAHLH (cellules Hela transfectées de manière stable avec un plasmide contenant le gène rapporteur XRE) et les cellules HepG2 (cellules hépatiques transfectées de manière transitoire avec un plasmide contenant le gène rapporteur XRE) ont été utilisés pour étudier l'activation d'AhR. Les résultats ont montré que GI et GII sont capables d'activer partiellement AhR. Les glycéollins peuvent également activer AhR dans les cellules cancéreuses mammaires ER-positives (cellules MCF-7) et ER-négatives (cellules MDA-MB-231). Dans tous les cas, l'effet de GII est globalement toujours plus fort que celui de GI.

Les résultats de l'analyse transcriptomique ont montré qu'environ 10% des gènes régulés par TCDD étaient également modifiés par GI et GII. On peut également observer des effets antagonistes ou synergiques de GI et GII lorsqu'ils sont co-traités avec TCDD. Ces gènes sont enrichis en gènes impliqués dans le métabolisme, la détoxification, l'apoptose et la communication cellulaire.

D'autre part, d'un point de vue phénotypique, GI et GII inhibent la migration des cellules cancéreuses mammaires triple-négatives MDA-MB-231. Ils modifient également l'expression des gènes codant des facteurs majeurs de migration et d'invasion cellulaire.

En résumé, les glycéollins présentent des effets agonistes et antagonistes partiels envers AhR et des effets anti-migration dans les cellules MDA-MB-231. Ces composés méritent d'être encore étudiés pour être utilisés comme traitement dans le CS triple négatif.

**PUBLICATION N°2 « Characterization of Glyceollins
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Their Role in Cell Migration »**

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Article

Characterization of Glyceollins as Novel Aryl Hydrocarbon Receptor Ligands and Their Role in Cell Migration

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Abstract: Recent studies strongly support the use of the aryl hydrocarbon receptor (AhR) as a therapeutic target in breast cancer. Glyceollins, a group of soybean phytoalexins, are known to exert therapeutic effects in chronic human diseases and also in cancer. To investigate the interaction between glyceollin I (GI), glyceollin II (GII) and AhR, a computational docking analysis, luciferase assays, immunofluorescence and transcriptome analyses were performed with different cancer cell lines. The docking experiments predicted that GI and GII can enter into the AhR binding pocket, but their interactions with the amino acids of the binding site differ, in part, from those interacting with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Both GI and GII were able to weakly and partially activate AhR, with GII being more potent. The results from the transcriptome assays showed that approximately 10% of the genes regulated by TCDD were also modified by both GI and GII, which could have either antagonistic or synergistic effects upon TCDD activation. In addition, we report here, on the basis of phenotype, that GI and GII inhibit the migration of triple-negative (ER-, PgR-, HER2NEU-) MDA-MB-231 breast cancer cells, and that they inhibit the expression of genes which code for important regulators of cell migration and invasion in cancer tissues. In conclusion, GI and GII are AhR ligands that should be further investigated to determine their usefulness in cancer treatments.

Keywords: glyceollins; aryl hydrocarbon receptor; breast cancer; cell migration

1. Introduction

Breast cancer (BC) is the most frequent cancer in women worldwide, with nearly 1.7 million new cases diagnosed in 2012 [1]. The latest 2019 estimates show that BC alone is expected to represent 30% of all new cancer cases diagnosed and to be the cause of 15% of cancer deaths in women [2]. Because of the progress in diagnosis and treatment, the BC death rate in woman declined by 40% from 1989 to 2016 [2]. However, BC is still the second leading cause of cancer death after lung and bronchus cancer. Particularly, for triple-negative BC, the most aggressive type, efficient treatment development remains a great challenge [3].

In addition, chemotherapy for advanced and metastatic cancers can induce severe toxicity, particularly in older patients [4]. Therefore, finding novel compounds with low toxicity that can be used as therapeutic adjuvants is of urgent necessity.

Glyceollins, including glyceollin I, II and III (GI, GII and GIII), are the most important phytoalexins present in soybeans exposed to certain fungi and some abiotic elicitors [5,6]. They serve as a protective system in plants through their antibacterial, antifungal and antinematode actions [7]. Recently, glyceollins have attracted positive attention because of their beneficial effects in human health [8,9]. In particular, glyceollins have shown anticancer effects in both estrogen receptor (ER)-dependent and ER-independent BC [8]. In the ER-dependent pathway, glyceollins have antiproliferative effects on mammary glands *in vivo*, on various ER-positive BC cells *in vitro* and on breast tumor cells in xenograft mice. These effects are mainly due to their antagonistic action on ERs [10–14]. In addition, glyceollins downregulate FOXM1, the key regulator of the cell cycle [10]. Glyceollins also suppress the phosphorylation of proteins known to cross-talk with ER signaling constituents, specifically p70S6K [15]. In the ER-independent pathway, glyceollins suppress the proliferation of ER-negative BC cells *in vitro* and *in vivo* [10,16]. Moreover, glyceollins inhibit tumor angiogenesis [17] and HIF-1 α synthesis and stability [18]. All of these published results support that glyceollins are promising candidates for BC treatment and should be further investigated.

The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor. The unliganded AhR is mainly localized in the cytosol, but is also found in the nucleoplasm among diverse cell types. Upon ligand binding, AhR is translocated from the cytosol to the nucleus, and binds to xenobiotic response elements (XREs) in the enhancer region of target genes. The AhR gene battery encodes cytochrome P450 isozymes (CYP1 and CYP2); various other drug-metabolizing enzymes; and proteins that control cell division, differentiation and apoptosis [19]. AhR was previously known as the mediator of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) toxicity [20] and of other environmental carcinogens. However, recent studies suggest that AhR plays important roles in normal physiology and pathophysiology, notably cancer [21–23]. The expression of AhR has been detected in a large number of tissues, such as those of the lung, liver, bladder, ovary and breast [24]. However, the expression level of AhR is rather heterogeneous, and varies greatly depending on the tissue, cell line and cancer category. Its involvement in tumorigenesis is not well established, but recent studies have shown that AhR can exhibit tumor-specific, pro-oncogenic and tumor suppressor-like functions. Thus, AhR antagonists or agonists can be potential treatments [22,25], depending on the specific type of tissue and ligand. An increasing number of studies have indicated that AhR plays a role in cell migration, notably in the epithelial–mesenchymal transition (EMT). Tsai et al. showed that AhR-regulated autophagy affects cancer cell EMT progression in lung cancer [26]. They also showed that an increase in AhR level can suppress histone deacetylase activity, which leads to inhibiting cell migration in gastric cancer [27]. As a result, the identification and characterization of natural molecules that can interact with and modulate AhR activity are expected to be particularly useful for the treatment of cancer.

From our previous transcriptome studies performed in ER-positive breast cancer cells treated with glyceollins, we found that the expression of several steroid and xenobiotic metabolism genes is markedly modified [10]. Since AhR is a key factor in the regulation of these genes, we wondered whether glyceollins are able to bind and activate AhR. Furthermore, the second aim of this study was to investigate the effects of glyceollins on breast cancer cell migration.

In this study, using *in vitro* and *in vivo* approaches, we showed that GI and GII have partial agonistic and antagonistic effects on AhR. Moreover, they exhibit anti-migration activity in the MDA-MB-231 mammary cancer cell line, which is correlated with diminished N-cadherin gene expression. In addition, both GI and GII modulated the expression of Chemokine (C-C motif) ligand 2 (CCL2) and PDZ-LIM domain protein 4 (PDLIM4) genes which code for important regulators of cell migration and invasion in cancer tissues.

2. Results

2.1. Docking at the Ligand-Binding Site and Experimental Interactions of AhR with Glyceollins

The Per-Arnt-Sim (PAS) domain of human AhR (residues 285–386) contains the full ligand binding domain (LBD) and the specificity of AhR [28]. To predict the 3D structure of the human AhR-LBD, we used the X-ray diffraction structure of the human PAS domain of the hypoxia-inducible factor 2 alpha (HIF-2 α) as a 3D coordinate template for homology modeling. We used the GOLD 5.2.2 program to perform docking simulations, which showed that the ligand-binding pocket of AhR has a cavity of 326 Å³, a finding in agreement with the results of Xing et al. [29]. In addition, our docking simulation for TCDD indicated the same orientation and position of the amino acids in the AhR pocket (Figure 1A). Furthermore, our model revealed several key amino acids (Phe 295, Leu 315, Gly 321, Leu 353, Met 340 and Val 381) that interact with TCDD and that were also reported in two previous studies as residues involved in the binding sites in human and mouse AhR [29,30]. It is interesting to note that, although both GI and GII can potentially be inserted into the AhR cavity, at a similar ligand binding pocket, a difference in the orientation of these molecules in the cavity compared to TCDD is observed. Consequently, different interactions with the cavity amino acids are indicated in the protein/ligand binding results (Figure 1A).

To determine whether glyceollins activate AhR, we first used HeLa cells stably expressing the XRE-luciferase plasmid containing an XRE sequence to generate HAHLH reporter cells [31]. The cells were incubated with increasing concentrations of glyceollin for 8 h and 16 h (Figure 1B). In the presence of GI, luciferase activity increased, but only after 16 h of incubation, while in the presence of GII, the progressive increase in luciferase activity was detected at both 8 h and 16 h of incubation. As hepatic cells are known to be a good cellular context for AhR function, we also used human hepatic HepG2 cells to analyze the glyceollin effect. HepG2 cells were transiently transfected with the XRE-luciferase reporter plasmid and incubated with different concentrations of GI and GII for 24 h (Figure 1C). Both glyceollins activated AhR weakly but significantly in the HepG2 cells treated with 1 μ M and 10 μ M GI or GII, compared to the cells treated with DMSO ($p < 0.05$). The effect of GI and GII at 10 μ M reached respectively 28% and 38% of the maximum transactivation efficiency observed with 1 nM TCDD. A representative image of labeled AhR in the HepG2 cells and its nuclear localization is shown in Figure 1D. The incubation of the cells with DMSO, TCDD (AhR reference ligand) and GI or GII at 10 μ M resulted in the different levels of the subcellular localization of AhR in the nucleus (Figure 1D). The lowest level of nuclear localization which was observed in the cells were treated with DMSO and GI, while those treated with TCDD and GII showed exclusively nuclear localization of AhR. This observation was confirmed by the quantification: the nucleus/cytoplasm fluorescence intensity clearly shows an increase in the nuclear labeling of AhR in the presence of TCDD and GII, but less in the presence of GI (Supplementary Figure S1). To avoid inaccurate conclusions on a false-negative result, a negative control without the primary antibody has also been run for all immunocytochemical assays (Figure 1D, right panels). Together, these results indicate that both glyceollins activated AhR in different cell lines, but GII was more effective in activating AhR.

In addition, in a previous study, we confirmed AhR activation by glyceollins *in vivo* by examining the expression of AhR-target genes CYP1A1 and CYP1B1 in the liver of mice treated with glyceollins [10]. As shown in Supplementary Figure S2, the expression levels of the two endogenous genes were significantly upregulated in the liver of the mice exposed to GI or GII.

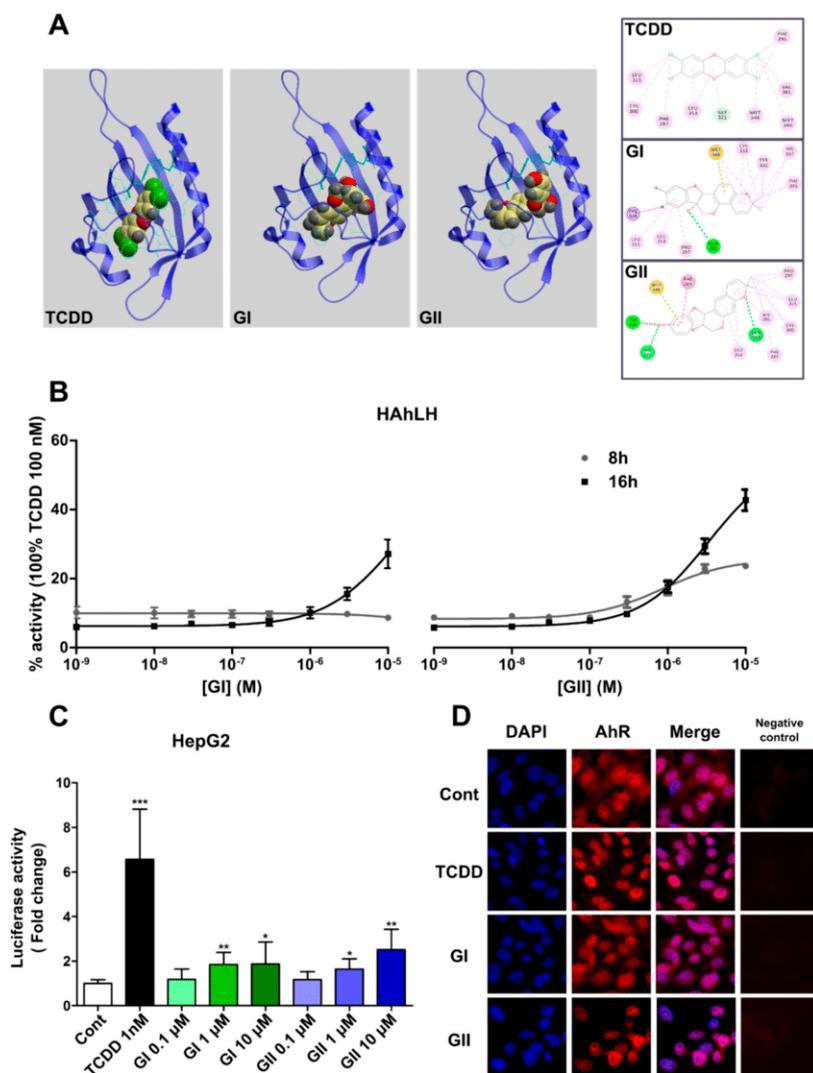


Figure 1. Glyceollin I (GI) and glyceollin II (GII) are aryl hydrocarbon receptor (AhR) ligands. (A) Docking analysis predicts that GI and GII can enter into the AhR binding pocket. *Left side:* Ribbon representation of the human AhR model with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), GI and GII docked in the binding cavity. The 3D model of the AhR binding pocket was obtained by structural homology modeling using hypoxia-inducible factor 2 alpha (HIF-2 α) as a template. *Right side:* Interactions of TCDD, GI and GII with the AhR binding cavity residues. (B) AhR is activated by GI and GII in HeLa cells stably transfected with the XRE₅(TnGCGTG)₃ luciferase reporter plasmid (HAhLH cells); these cells were treated with different concentrations of GI (*left side*) and GII (*right side*) for two different incubation times (8 h and 16 h). The results are expressed as the percent of luciferase activity obtained with 100 nM TCDD. The experiment was done three times in quadruplicate. (C) AhR is activated by GI and GII in human HepG2 hepatoma cells expressing endogenous AhR. Cells were transiently transfected with an XRE \times 3-luciferase reporter plasmid and a CMV- β -galactosidase plasmid as a control for transfection efficiency. These cells were then treated with 0.1% (*v/v*) dimethyl sulfoxide (DMSO) (white) to serve as the control (Cont), TCDD (black) at 1 nM and different doses of GI (green) or GII (blue) for 24 h. The results are expressed as the fold change in luciferase activity compared with that in the control and are represented as the mean \pm SD. The experiment was done four times in triplicate. Statistical analyses were performed with one-way analysis (ANOVA) followed by Dunnett's post hoc test. * *p*-value < 0.05, ** *p*-value < 0.01, *** *p*-value < 0.001 are considered to be significantly different with the control group. (D) Subcellular localization of AhR in HepG2 cells treated with DMSO, TCDD at 1 nM, GI or GII at 10 μ M for 24 h, as examined by immunofluorescence. The negative controls represent immunocytochemical assays without the primary antibody (right panels).

2.2. AhR Activation by Glyceollins in Breast Cancer Cells

As the cross-talk between AhR and ER α has been well documented at the transcriptional level [32], we investigated the activation of the AhR reporter gene by the glyceollins in both ER-positive MCF-7 and ER-negative MDA-MB-231 breast cancer cells (Figure 2A,B). As shown by Western blotting, AhR was expressed in both cell lines, but ER was expressed only in the MCF-7 cells (Figure 2C). In the HepG2 cells, the standard model to study AhR, GI and GII at 1 μ M or higher concentrations activated AhR (Figure 1C). Thus, we started by this dose to study the effect of the glyceollins on other cell lines. In the MCF-7 cells, GII activated AhR at a concentration of 1 μ M, and this effect was stronger at 10 μ M. This effect was reversed by the addition of CH 223191, an AhR inhibitor, to the cells. In the MDA-MB-231 cells, at 10 μ M, GI but not GII-activated AhR. In these cells, AhR activation by TCDD was also much lower than it was in the MCF-7 cells (Figure 2A). Therefore, the slight ligand-dependent activation of AhR observed in the MDA-MB-231 cells may be due to the strong AhR expression in this cell line compared to that in the MCF-7 cell line.

We also used a luciferase assay to examine the activation of XRE-CYP1A1 promoter activation in the MCF-7 cells. The CYP1A1 promoter luciferase activity was increased in the presence of both GI and GII at 10 μ M (Supplementary Figure S3), but GII appeared to increase the luciferase more efficiently. Next, the recruitment of AhR to AhR-responsive regions to CYP1A1 and CYP1B1 was examined using ChIP-PCR. As shown in Figure 2D, both TCDD and GII, but not GI, increased AhR binding to the XRE in the MCF-7 and MDA-MB-231 cells. In the MCF-7 cells, AhR recruitment by GII appeared to be lower than that of TCDD, but in the MDA-MB-231 cells, GII and TCDD recruited AhR at the same level. The results support that GII induces the binding of AhR to XRE-containing regions in AhR target genes via AhR and regulates the expression of these genes, while the effect of GI is weaker. In summary, glyceollins activate AhR in both ER-positive and ER-negative breast cancer cell lines, with GII having a greater overall effect than GI.

2.3. Genome-Wide Analysis of the Glyceollin Effects

To thoroughly understand the actions of glyceollins, and in particular, to assess the effects of glyceollins on AhR-target genes, we first performed a transcriptome analysis of HepG2 cells treated with GI or GII either alone or in combination with TCDD. The data were deposited in gene expression omnibus (GEO) (GSE141711) and in TOXsIgN. After statistical filtration, 565 differentially expressed genes were selected (Figure 3A) and further classified into nine expression clusters (termed C1–C9) (Figure 3B). Briefly, the C1–C2 clusters were associated with genes showing additive effects of the glyceollins and TCDD compared to the effects of the control. In cluster C1, the treatment of cells with GI, GII or TCDD alone slightly upregulated gene expression, while cotreatments showed stronger effects. The same effects were observed in cluster C2 in the downregulation direction. The C3–C4 clusters included genes for which GI and GII antagonized the TCDD effect in terms of transcript abundance. Indeed, treatment with TCDD alone seemed to significantly increase or decrease the expression of the genes belonging to the C3 and C4 clusters, respectively, whereas cotreatment with GI or GII seemed to reduce this transcriptional effect. The C5–C7 clusters included genes specifically dysregulated by GI and GII. The C8 cluster corresponded to genes specifically dysregulated by TCDD. Finally, C9 included genes downregulated under all conditions. The same strategy was also used to evaluate the effects in the MCF-7 and MDA-MB-231 cells. We identified 237 and 83 genes showing significant differential expression in the MCF-7 and MDA-MB-231 cells, respectively (Supplementary Figure S4). Among the differentially expressed genes in HepG2 cells, 160, 187 and 202 genes were differentially expressed after exposure to GI, GII and TCDD, respectively (Figure 3A), and 35 genes were found to be dysregulated by both TCDD and the glyceollins (Figure 3C). The functional analysis of this subset of genes revealed that they were significantly enriched in genes involved in metabolism and detoxification, apoptosis and cell communication and with extracellular exosomes (Figure 3D).

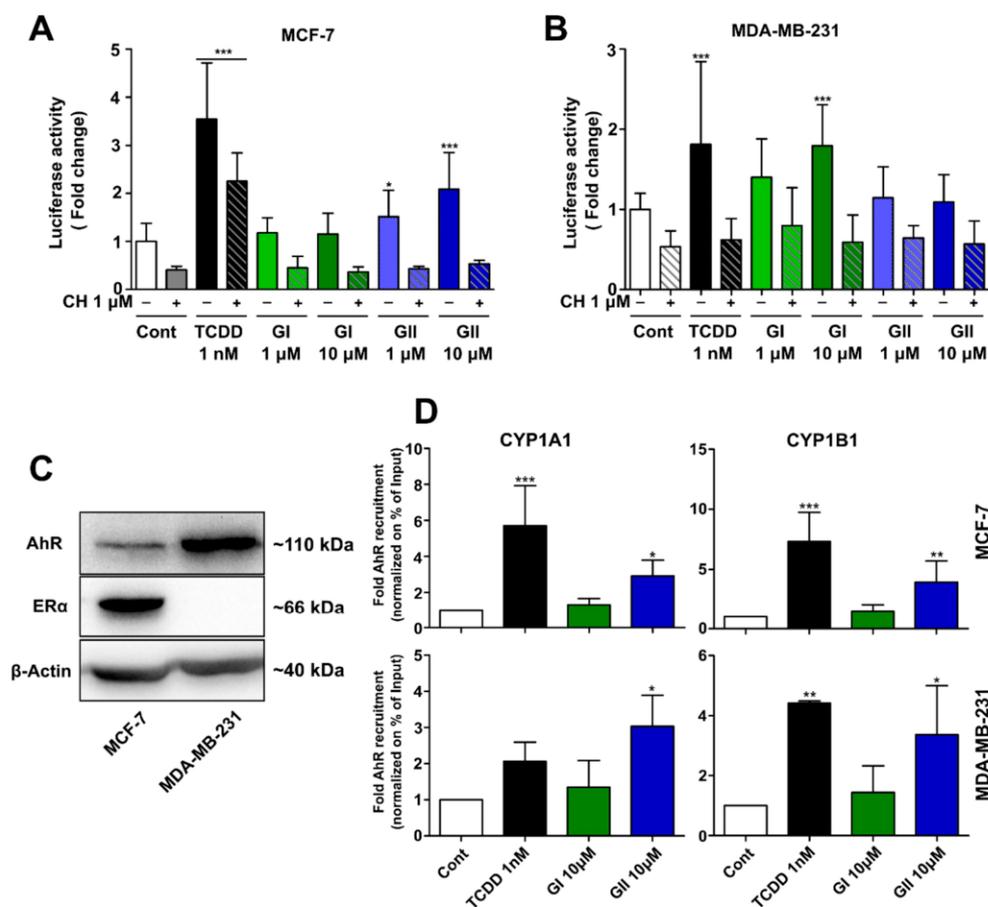


Figure 2. Effects of the glyceollins on aryl hydrocarbon receptor (AhR) signaling in estrogen receptor (ER)-positive MCF-7 and ER-negative MDA-MB-231 breast cancer cells. **(A,B)** AhR-mediated transcriptional activity was analyzed by transfecting cells with an XRE \times 3-luciferase reporter plasmid and a CMV- β -galactosidase plasmid as a control for transfection efficiency. MCF-7 cells **(A)** and MDA-MB-231 cells **(B)** were treated for 24 h with 0.1% (*v/v*) DMSO (white) to serve as the control (Cont), 1 nM 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (black) or two different concentrations of glyceollin I (GI) (green) or glyceollin II (GII) (blue), alone or in combination with 1 μ M CH 223191 (CH), an AhR antagonist. The results are expressed as the fold change in luciferase activity compared with that in the control, and are represented as the mean \pm standard deviation (SD). The experiment was done at least three times in triplicates. **(C)** Equal amounts of protein extracts from the MCF-7 and MDA-MB-231 cells were analyzed by Western blotting as revealed by the binding of specific antibodies to AhR, ER α and β -actin. The molecular weight at kDa for each protein is indicated. **(D)** Recruitment of AhR to the chromatin of CYP1A1 and CYP1B1 genes through the AhR binding site in response to TCDD, GI and GII. MCF-7 and MDA-MB-231 cells were treated with 0.1% (*v/v*) DMSO (white) to serve as the control, TCDD (black) at 1 nM, GI (green) or GII (blue) at 10 μ M for 1 h prior to chromatin cross-linking and chromatin immunoprecipitation (ChIP) with the AhR antibody. CYP1A1 and CYP1B1 gene expression in the XRE-containing regions was then examined by real-time polymerase chain reaction (PCR). The DNA sequences for each AhR-binding site tested are presented in the methods section. Receptor recruitment is presented as percent of input. The results are expressed as the fold change compared with the level of the control, and are presented as the mean \pm SD. The experiment was done seven times in MCF-7 cells and three times in MDA-MB-231 cells in triplicate. Statistical analyses were performed with one-way ANOVA followed by Dunnett's post hoc test. * *p*-value < 0.05, ** *p*-value < 0.01, *** *p*-value < 0.001 are considered to be significantly different with the control group.

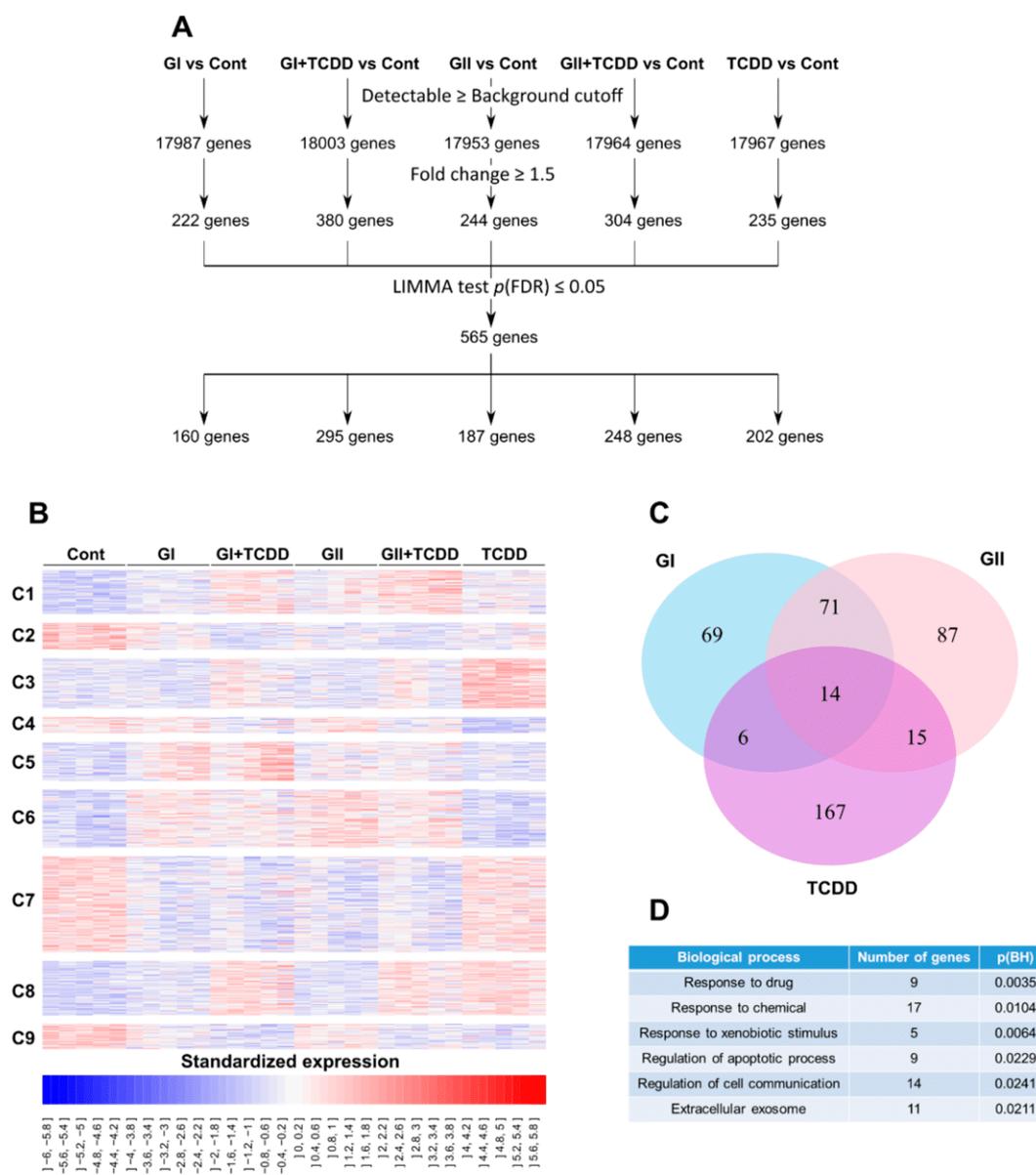


Figure 3. Transcriptome analysis, selection and clustering of differentially expressed genes in the HepG2 cells. HepG2 cells were treated with 0.1% (*v/v*) DMSO to serve as the control (Cont), 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) at 1 nM, glyceollin I (GI) or glyceollin II (GII) at 10 μ M alone or in combination with TCDD for 24 h. Total RNA was extracted, labeled, reverse transcribed and sequenced. (A) The differentially expressed genes for each treatment were selected in comparison to the control levels. Then all probes with an intensity signal greater than the overall median and a fold change ≥ 1.5 were selected. The genes were then grouped together and subjected to a LIMMA test; and only those probes with a *p*-value ≤ 0.05 were retained, resulting in a total of 565 differentially expressed genes. The number of differentially expressed genes in each treatment is indicated. (B) A total of 565 differentially expressed genes were clustered into nine clusters on the basis of their expression patterns. (C) Venn diagram created from the list of differentially expressed genes obtained from comparisons with the control and GI (blue), GII (pink) and TCDD (purple). (D) Main biological processes that the differentially expressed genes by TCDD and GI or GII involve in.

2.4. Glyceollin Effects on AhR Target Gene Expression

Next, we validated the effects of the glyceollins on CYP1A1 and CYP1B1 gene expression by real-time PCR in the different cell lines. The cells were treated with each glyceollin alone or in

combination with the AhR antagonist, CH 223191 (Figure 4A,C,E,G,I) or with TCDD (Figure 4B,D,F,H,J). First, in the HepG2 cells, both GI and GII added alone increased the CYP1A1 expression (Figure 4A). Interestingly, both GI and GII significantly decreased the upregulation of these genes induced by TCDD (Figure 4B). It should be noted that the CYP1B1 gene is not expressed in HepG2 cells [33]. In the MCF-7 cells, GI and GII dramatically increased the expression of both CYP1A1 and CYP1B1 genes, especially CYP1A1 (Figure 4C–F). GI and GII also decreased the TCDD upregulation of these genes. However, in the MDA-MB-231 cells, the gene expression was also significantly changed when treated with each glyceollin alone, but with less remarkable fold change (Figure 4G,I). This result was consistent with that of the luciferase assay in the MDA-MB-231 cells. Nevertheless, in combination with TCDD, each glyceollin treatment significantly decreased CYP1A1 gene expression compared to that of TCDD treatment alone. Overall, the results show that activations of CYP1A1 and CYP1B1 are mediated by AhR, and that glyceollins have a stimulating effect when treated alone, and have an inhibitory effect when combined with TCDD on AhR-target gene expression.

2.5. Glyceollin Effect on Cell Migration

As several recent studies have indicated that AhR signaling is profoundly involved in cell migration [34,35], we tested the effect of glyceollins on cell migration using an IncuCyte ZOOM[®] 96-Well Scratch Wound cell migration assay. We used the triple-negative BC MDA-MB-231 cells, which are known to be highly invasive. As shown in Figure 5, GI and GII significantly attenuate cell migration within 4 h of treatment. This effect was more obvious at 36 h of treatment, both in terms of wound width and wound confluence (Figure 5C,D). It is noteworthy that the statistical analysis indicated that the effect of GII is stronger than that of GI. Consistent with these findings, we examined the effects of GI and GII on the expression of N-cadherin (CDH2), Chemokine (C-C motif) ligand 2 (CCL2) and PDZ-LIM domain protein 4 (PDLIM4) genes which code for factors that are essential regulators of cell migration [36–38]. In fact, N-cadherin is an active inducer of the metastatic behavior of tumor cells that directly modulates cell–cell adhesion [36]. The chemokine CCL2 mediates the prometastatic effects of dysadherin in cancer cells, notably in ER-negative MDA-MB-231 breast cancer cells [37]. Otherwise, the expression of PDLIM4 is often downregulated in the advanced tumor stage of cancer tissues and lymph node metastasis [38]. Interestingly, we observed that the effects of GI and GII on cell migration is associated with a reduction in N-cadherin gene expression. Indeed, GII decreased N-cadherin gene expression more than 2-fold, and GI also decreased, but not significantly (Figure 5F). Otherwise, both GI and GII significantly reduced CCL2 expression and enhanced PDLIM4 expression (Figure 5F). It is also noteworthy that glyceollins also have antiproliferative effects in these cell lines (Supplementary Figure S5), as reported previously [16]. However, the antiproliferative effect on MDA-MB-231 cells require a longer time (at least 72 h of treatment) whereas the anti-migration effect was observed as early as 4 h of treatment.

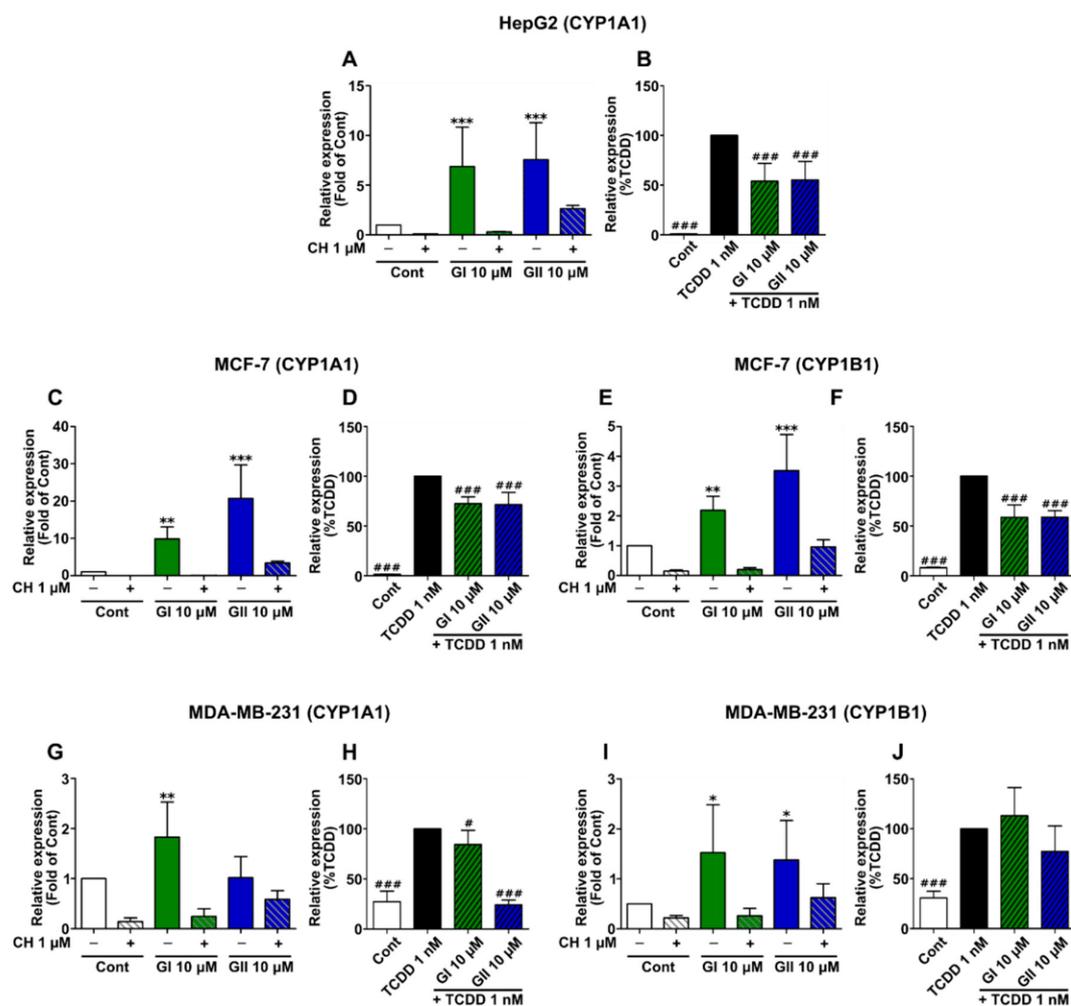


Figure 4. Partial agonistic and antagonistic effects of glyceollins in AhR target gene expression in different cell lines. HepG2 (A,B), MCF-7 (C–F) and MDA-MB-231 (G–J) cells were treated with 0.1% (*v/v*) DMSO to serve as the control (Cont) and 1 nM 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), 10 μ M glyceollin I (GI) and 10 μ M glyceollin (GII) alone or in combination with 1 μ M CH 223191 (CH), an AhR antagonist or with TCDD, for 24 h. The relative expression of CYP1A1 (A–D,G,H) and CYP1B1 (E,F,I,J) was assessed by real-time PCR and normalized to the expression of the housekeeping gene GAPDH and TBP. Notably, HepG2 cells do not express CYP1B1, while MCF-7 and MDA-MB-231 cells express both genes. The results were expressed as the fold change relative to the level of the DMSO-treated cells (Cont) to show the agonistic effect of the glyceollins (panels A,C,E,G,I) or relative to TCDD-treated cells to show the partial antagonistic effect of the glyceollins (panels B,D,F,H,J). The results are expressed as the mean \pm SD. The experiment was done at least three times in each cell line in triplicate. Statistical analyses were performed with one-way ANOVA followed by Dunnett’s post hoc test. * *p*-value < 0.05, ** *p*-value < 0.01, *** *p*-value < 0.001 are considered to be significantly different with the control group. # *p*-value < 0.01, ### *p*-value < 0.01, #### *p*-value < 0.001 are considered to be significantly different with the TCDD group.

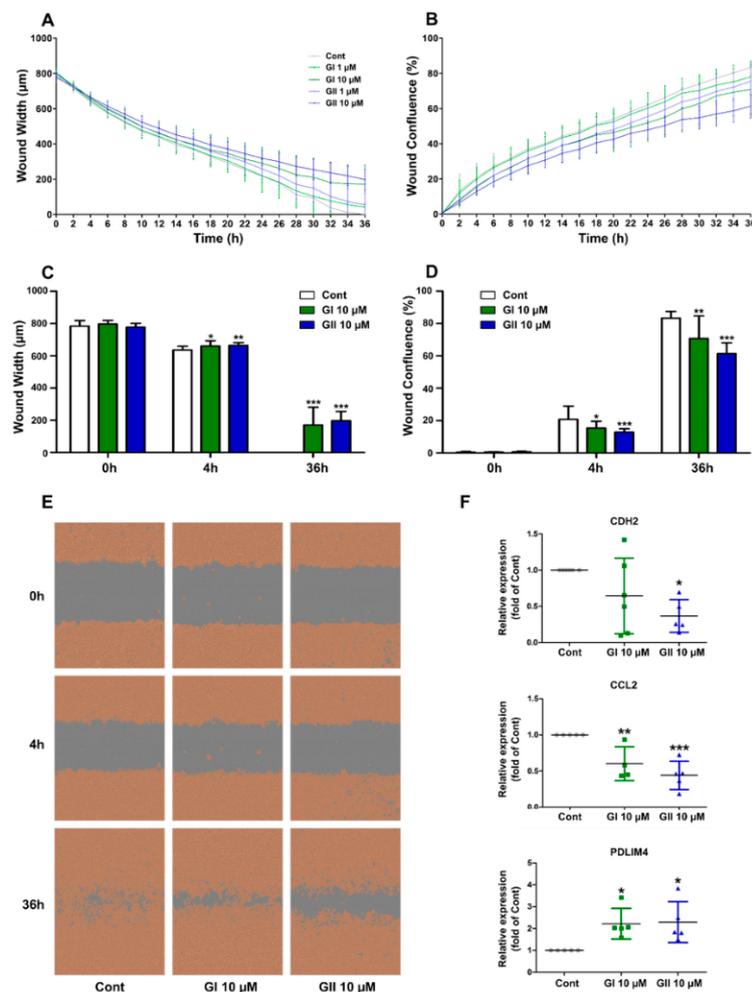


Figure 5. Effect of glyceollins on cell migration. MDA-MB-231 cells were cultivated in a 96-well plate until confluence, and then, a wound was created. After being wounded, cells were treated with 0.1% (*v/v*) DMSO (Cont), glyceollin I (GI) or glyceollin II (GII) at two different concentrations. Then, the cell plate was placed in an IncuCyte ZOOM[®] instrument and scanned every 2 h. Wound width and wound confluence were calculated using IncuCyte ZOOM[®] software. Wound width was measured as the average distance between the edges of the scratch wound mask in each line of resolution within an image. Wound confluence measures the cell confluence in only the wound region, which is determined using the borders of the wound region in the first image. (A) Kinetics of wound width over 36 h. (B) Kinetics of wound confluence over 36 h. Each point represents the mean \pm SD of data of three independent experiments in quintuplicate. (C) Wound width at 0 h, 4 h and 36 h post-wounding. (D) Wound confluence at 0 h, 4 h and 36 h post-wounding. At each time point, statistical analyses were performed with one-way ANOVA followed by Dunnett's post hoc test. * *p*-value < 0.05, ** *p*-value < 0.01, *** *p*-value < 0.001 are considered to be significantly different with the control group. (E) Representative images taken at 0 h, 4 h and 36 h post-wounding and 0.1% (*v/v*) DMSO, GI 10 μ M and GII 10 μ M treatments. Confluence masks in orange as merged with the initial phase images. The confluence mask overlay indicates areas of the image that are occupied by cells. (F) The relative gene expression of N-cadherin (CDH2), Chemokine (C-C motif) ligand 2 (CCL2) and the PDZ-LIM domain protein 4 (PDLIM4) after 24 h of 0.1% (*v/v*) DMSO (Cont), GI and GII at 10 μ M treatment, as assessed by real-time PCR. The results were normalized to the expression of the housekeeping genes GAPDH and TBP, and presented as the fold change compared to level of the control. Each point reflects an independent experiment. Statistical analyses were performed with one-way ANOVA followed by Dunnett's post hoc test. * *p*-value < 0.05, ** *p*-value < 0.01, *** *p*-value < 0.001 is considered to be significantly different with the control group.

3. Discussion

To date, although the role of AhR in cancer remains unclear, and some results are contradictory, many studies have shown the anticancer effects of AhR ligands in breast cancer. Flavipin, an AhR agonist, suppresses the migration and invasion of BC cells [34]. Genistein can confer sensitivity to triple-negative breast cancer for antiestrogen therapy by preventing and reversing AhR-dependent BRCA1 hypermethylation [39]. Aminoflavone, another AhR ligand, inhibits the proliferation of tamoxifen-resistant cells by suppressing α 6-integrin-Src-Akt signaling [40]. Raloxifene induces apoptosis mediated by AhR in ER-negative BC cells [41]. Omeprazole, a proton pump inhibitor, activates AhR and decreases breast cancer cell migration and invasion [35]. These pieces of evidence show that AhR can be a therapeutic target in breast cancer. Glyceollins, a group of phytoalexins isolated from soybeans, have attracted attention since 2000, because they exert numerous effects on human functions and diseases, notably cancer. Since the discovery of their properties, many studies have elucidated their mechanism of anticancer action, particularly in breast cancer [8]. However, this study is the first to show the effect of glyceollins on AhR signaling and on triple-negative BC cell migration.

To thoroughly understand the interaction between glyceollins and AhR, we used different cell models but also examined the activation of AhR, *in vivo*, by analyzing the expression of CYP1A1 and CYP1B1 in mouse livers. First, we measured the activation of the XRE-luciferase reporter gene in stably transfected HeLa cells and transiently transfected HepG2 cells. These two cell models were used widely in previous studies investigating AhR activation [31,42,43]. In our study, we used these cells as reference models to determine the effect of glyceollins on AhR, which enabled us to choose the appropriate doses for further investigations. Then, we used both ER-positive MCF-7 and ER-negative MDA-MB-231 breast cancer cells. Since it was well demonstrated that there is cross-talk between ER and AhR [32,44], and that glyceollins have effects on ER action [10], the use of these cell contexts makes for a complete study. Finally, we studied glyceollin effects in cell migration only in the MDA-MB-231 triple-negative breast cancer cells, because cells of this lineage represent the most metastatic type of breast cancer [45]. Moreover, previous studies showed that AhR is an important target of cell migration in triple-negative BC [34,35].

The findings of our simulation model of the interaction of AhR with TCDD, used as a reference ligand, were consistent with two published studies on human and mouse AhR [29,30]. Our docking experiments showed that both GI and GII can enter the AhR ligand binding pocket, although GI and GII have the larger structure than TCDD. While our model predicts the interaction of GI and GII in the AhR cavity, we however observed a difference in the orientation of these molecules in the binding pocket compared to TCDD. Therefore, the amino acids involved in protein/ligand binding are substantially different (Figure 1A). These variations in orientation and residue interactions predict that AhR can adopt a different conformation when activated with glyceollins compared to TCDD. It also suggests that glyceollins effects, mediated by AhR, could induce distinct biological responses compared to those induced by TCDD.

This could explain the difference in response between GI, GII and TCDD observed in the luciferase assays. In both HeLa and HepG2 cells, GI and GII activate AhR, but their action is weaker than that of TCDD (Figure 1). AhR activation by glyceollins was also observed in BC cell lines (Figure 2). Then, at the genomic level, we studied glyceollin treatment both alone and in combination with TCDD. The results from the transcriptome analysis (Figure 3) showed that glyceollins can modify many of the same genes as TCDD modifies. However, glyceollins can both fortify and antagonize the effects of TCDD. Among the differentially expressed genes found for both TCDD and the glyceollins, a significant number was involved in metabolic and detoxification, apoptosis, cell communication and extracellular exosomes.

We chose two AhR-target genes, CYP1A1 and CYP1B1, to validate these findings by RT-PCR. While the glyceollins alone exerted a weak agonistic effect on AhR signaling, their cotreatment with TCDD led to an antagonistic effect that counteracted the effect of TCDD in all three cell lines investigated. This finding suggests that glyceollins are natural molecules that can have partial agonistic and antagonistic

effects on AhR signaling. However, the effects of these glyceollins in MDA-MB-231 cells, at the gene expression level and at the ligand-receptor level, were less remarkable than they were in other cell lines, even compared to the expression of the positive control TCDD. This may be due to the high expression of AhR in these cells, as shown in our experiment (Figure 2B) and in other studies [44,46]. In addition, the high level of endogenous AhR ligands, such as the tryptophan derivatives in MDA-MB-231 cells [46], may have led to ligand competition. Since CYP gene activation through AhR, particularly CYP1A1, is mediated by the toxicity of TCDD [47], with a partial antagonistic effect, glyceollins can be candidates for the detoxification of TCDD and other AhR-active toxicants. Some natural compounds, such as genistein and resveratrol, have also shown to antagonize the effect of TCDD [48,49].

Cancer metastasis is a leading cause of cancer-related mortality [50]. Metastasis is facilitated by the EMT process, which is marked by the loss of epithelial cadherin (E-cadherin) expression and the concomitant upregulation or de novo expression of neutral cadherin (N-cadherin) [36]. Thus, N-cadherin expression is a signal of epithelial-to-mesenchymal transition, leading to the acquisition of an aggressive tumor phenotype [36]. MDA-MB-231 cells, the most aggressive type of breast cancer, show typical EMT with high expression of N-cadherin. However, the role of AhR in EMT remains contradictory. Murray et al. suggested that an increase in AhR activity leads to the initiation of EMT and facilitates tumor cell migration, invasiveness and metastasis [23]. However, Tsai et al. showed that a low expression of AhR contributed to high cell migration by decreasing E-cadherin and increasing vimentin [26]. In our study, GI and GII showed an antimigration effect as early as 4 h after treatment, and they decreased N-cadherin gene expression after 24 h of treatment. In addition, our results showed that GI and GII significantly modulate the expression of two potent regulators of cell migration, the chemokine CCL2 and PDLIM4 (Figure 5). These results, as well as the evidence from our transcriptome analyses, revealed glyceollin effects on the expression of genes involved in cell communication, a finding consistent with the studies by Carriere et al. [51], who showed that GI reverses EMT in letrozole-resistant breast cancer cells, and Rhodes et al. [16], who reported that glyceollins alter microRNA and proteome expression in EMT, cell migration and invasion. Furthermore, in human arterial smooth muscle cells, glyceollins have also been shown to inhibit cell migration by blocking platelet-derived growth factor [52].

In summary, GI and GII show partial agonistic and antagonistic effects toward AhR and antimigration effects in MDA-MB-231 cells. Glyceollins merit further investigation for use as a triple-negative BC treatment.

4. Materials and Methods

4.1. Computer Simulation of AhR Ligand Interactions (Docking)

Homology modeling was used to predict the 3D structure of the LBD in the human AhR (hAhR) at amino acids 285–386 [28]. The X-ray diffraction structure, available from the Protein Data Bank, of the human PAS domain in hypoxia-inducible factor 2 α , HIF-2 α (4ZP4b) was used as a 3D coordinate template for the homology modeling of the hAhR-ligand binding domain (LBD). The 3D models of hAhR-LBD were generated using RaptorX [53]. Identification and characterization of internal cavities in the model structure were performed using CAVER 3.0 software [54], a program that enables the identification and calculation of Connolly's molecular surface and volume [55] for all pockets and cavities in a protein structure. The program ranks the cavities by size, with the largest cavity usually being the binding site.

The optimized 3D conformers of GI, GII and TCDD were downloaded from the PubChem Open Chemistry Database (<https://pubchem.ncbi.nlm.nih.gov>). Docking was carried out using GOLD 5.2.2 (<https://www.ccdc.cam.ac.uk/solutions/csd-discovery/components/gold/>). GOLD optimizes the fitness score by using a genetic algorithm. The GOLD nuclear hormone receptor template was used with the ChemPLP scoring function and default parameters [56]. The hAhR model ligand-binding site of the optimized 3D protein model was defined as the residues with at least one heavy atom within 10 Å

(standard default) from the cognate ligand placement. Receptor hydrogens were added, and all atom valency levels are satisfied such that atoms can be properly identified. For optimal accuracy, the GA setting was set to the “very flexible” parameter. The constraint “never dock a ligand when a constraint is physically impossible” was applied. Ten dockings for each ligand are performed, and the runs were stopped for three solutions that were within 1.5 Å RMSD (default setting).

4.2. Cell Cultures and Reagents

HepG2, HeLa, MCF-7 and MDA-MB-231 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen, Waltham, MA, USA) supplemented with 4,5 g/L glucose and nonessential amino acids (NEAA, Invitrogen, Waltham, MA, USA), antibiotics penicillin/streptomycin (Invitrogen, Waltham, MA, USA) and 10% fetal bovine serum (FBS, Biowest, Nuaillé, France). All the cells were cultured at 37 °C under 5% CO₂. Before treatment, the cells were cultured for at least 24 h in steroids and serum-free DMEM without phenol red and with 1.5% charcoal/dextran-stripping FBS (Biowest, Nuaillé, France). TCDD was purchased from Interchim (Montluçon, France). CH 223191 was purchased from Sigma-Aldrich (St Louis, MO, USA). GI and GII were chemically synthesized by HPC Pharma as described in our previous study [10]. The purity was determined to be 98% and 99% for GI and GII, respectively.

4.3. Immunofluorescence

HepG2 cells were grown on 10-mm-diameter coverslips in 24-well plates. After serum and steroid deprivation, the cells were treated with 0.1% (*v/v*) DMSO, 10^{−9} M TCDD, 10^{−5} M GI and 10^{−5} M GII for 24 h. After treatment, the cells were fixed with 4% formaldehyde solution/neutral buffered (Sigma-Aldrich, St Louis, MO, USA) for 15 min and then permeabilized in PBS/3% Triton X-100 for 10 min. Next, the cells were unmasked with 50 mM Tris-HCl pH 9.5 at 80 °C for 30 min. The cells were incubated overnight at 4 °C with a primary rabbit polyclonal antibody to AhR (sc-5579, Santa Cruz, Santa Cruz, CA, USA) diluted at 1:250 and then a secondary goat anti-rabbit Alexa Fluor 594 antibody for one hour (dilution 1:1000). The coverslips were mounted with Duolink[®] in-situ mounting medium with 4′,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, St Louis, MO, USA) before microscopy analysis.

4.4. Luciferase Assay

50,000 cells/wells of HepG2, MCF-7 and MDA-MB-231 cells were seeded in 24-well plates. After 24 h, the cells were incubated with serum and steroid-deprived medium. The cells were transfected with JetPEI (Polyplus-transfection) overnight with 100 ng of an XRE-luciferase vector.

This vector encodes luciferase under the control of three XREs. As a control for the efficacy of the transfection, the CMV-β galactosidase vector was used. The following day, the cells were treated with TCDD 10^{−9} M or different doses of GI or GII. CH 223191 10^{−6} M was used as an AhR inhibitor. Then the reporter lysis buffer (Promega, Madison, WI, USA) was used to lyse the cells, and the luciferase activity was measured with a commercially luciferase assay system (Promega, Madison, WI, USA). The luciferase activity was normalized with β galactosidase activity. Data were then represented in a fold change of the control condition.

The HAHLH reporter cells were HeLa cells stably transfected with 5(TnGCGTG)₃-tata-luciferase-Luc-hygromycin plasmid in which the luciferase reporter gene was controlled by XRE 5(TnGCGTG)₃. The HAHLH cells were seeded at a density of 25,000 cells/well in 96-well culture plates in DMEM F12 without phenol red and supplemented with 5% dextran-coated charcoal-treated fetal calf serum. The HAHLH cells were incubated for 8 h and 16 h with 0.1% (*v/v*) DMSO, TCDD 100 nM or different concentrations of glyceollins, and then, the luciferase activity was measured as previously described [31].

4.5. Protein Extraction and Western Blotting

The MCF-7 and MDA-MB-231 cells were lysed in NP-40 lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Ninodet P40, 0.5% sodium deoxycholate and 0.1% SDS] containing protease inhibitor (Sigma-Aldrich, St Louis, MO, USA), vortexed strongly every 5 min for 30 min and then centrifuged at 12,000 rpm for 30 min at 4 °C. The protein concentrations were measured using a DC™ protein assay kit (Bio-Rad, Hercules, CA, USA). Protein extracts (25 µg/µL) were prepared in Laemmli buffer. The protein extracts were denatured for 10 min at 95 °C, separated on 8.5% SDS polyacrylamide gels, and transferred to polyvinylidene difluoride membrane (Millipore, Darmstadt, Germany). The proteins were then probed with specific antibodies. The following antibodies were used: anti-ERα HC20 (sc543, Santa Cruz, Santa Cruz, CA, USA), anti-AhR A-3 monoclonal mouse (Santa Cruz, sc-133088) and anti-β-actin C20 (sc8432, Santa Cruz, Santa Cruz, CA, USA), which served as a control for protein level. An enhanced chemiluminescence system (Immune-Star, Bio-Rad, Hercules, CA, USA) was used to detect the immunocomplexes.

4.6. Chromatin Immunoprecipitation (ChIP) Assays

The MCF-7 and MDA-MB-231 cells were treated with DMSO (Cont), and 1 nM TCDD or 10 µM glyceollin I or II for 1 h, washed twice with PBS and cross-linked for 10 min with 1.5% formaldehyde (Sigma-Aldrich, St Louis, MO, USA). The cross-linking reaction was stopped with 0.1 M glycine (Euromedex, Souffelweyersheim, France) for 1 min, and the cells were washed twice with cold PBS, scraped into 500 µL of cold PBS, spun 2 min at 3000 rpm and maintained at –80 °C. Then, the cells were lysed with lysis buffer [10 mM EDTA, 50 mM Tris-HCl (pH 8.1), 0.5% Empigen BB, and 1% SDS] and supplemented with a protease inhibitor (Roche) immediately before use. The cell lysates containing the cross-linked chromatin complexes were sonicated and immunoprecipitated with AhR antibody H-211 (sc-5579, Santa Cruz, Santa Cruz, CA, USA). The DNA was purified on NucleoSpin columns (Macherey-Nagel, Duren, Germany) using NTB buffer. ChIP DNA was used for real-time PCR. The primers used to amplify the AhR binding regions of the genes of interest are listed in Table 1.

Table 1. Gene names and primer sequences used in this study.

Experiments	Gene Name and Symbol	Forward Primer	Reverse Primer
Real-time PCR	Cytochrome P450 family 1 subfamily A member 1 (CYP1A1)	GCTGACTTCATCCCTATTCCTCG	TTTTGTAGTGCTCCTTGACCATCT
	Cytochrome P450 family 1 subfamily B member 1 (CYP1B1)	GCAACTTCAGCAACTTCATC	ATAAAGGCGTCCATCATGTC
	N-Cadherin (CDH2)	GACAATGCCCTCAAGTGTT	CCATTAAGCCGAGTGATGGT
	Chemokine (C-C motif) ligand 2 (CCL2)	AAGATCTCAGTGCAGAGGCTCG	TTGCTTGCCAGGTGGTCCAT
	PDZ-LIM domain protein 4 (PDLIM4)	CAAGGCACGGGACAAGCTCTAC	AGCAGGGACCTTAAGAAGCAG
	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG
	TATA box-binding protein (TBP)	TGCACAGGAGCCAAGAGTGAA	CACATCACAGCTCCCCACCA
ChIP-PCR	Cytochrome P450 family 1 subfamily A member 1 (CYP1A1)	GCGCGAACCTCAGCTAGT	TTCCCGGGTTACTGAGTC
	Cytochrome P450 family 1 subfamily B member 1 (CYP1B1)	ATATGACTGGAGCCGACTTTTCC	GGCGAACTTATCGGGTTGA

4.7. RNA Extraction and Real-Time PCR

The MCF-7, MDA-MB-231 and HepG2 cells (350,000; 250,000; and 500,000 cells/well, respectively) were plated in 6-well plates. After 24 h of serum and steroid deprivation, the cells were treated with

0.1% (*v/v*) DMSO to serve as a control, with TCDD 10^{-9} M or with different concentrations of GI or GII. Livers from ovariectomized RjOrl SWISS female mice were obtained from our previous study [10]. The total RNAs were obtained using a mini RNeasy (Qiagen, Hilden, Germany) kit in accordance with the manufacturer's instructions. Then, using an M-MLV RT (Invitrogen, Waltham, MA, USA) kit, the RNAs were reverse-transcribed according to the manufacturer's instructions. For real-time PCR, 5 ng of cDNA was employed with 150 nM primers (Table 1) and iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, USA). Real-time PCR was carried out in a CFX 384 apparatus, and the data were analyzed with CFX Manager software (Bio-Rad, Hercules, CA, USA).

4.8. Cell Migration Assays

The MDA-MB-231 cells (20,000 cells/well) were plated in 96-well plates and cultured until confluent. Then, wounds were created in all wells following the 96-well WoundMaker™ procedure (EssenBioScience, Ann Arbor, MI, USA). Then, all the wells were washed with culture medium and treatments were added. The cell plates were placed in an IncuCyte ZOOM® instrument and scanned every 2 h. The wound width and wound confluence were calculated using IncuCyte ZOOM® software. The wound width represents the average distance (μm) between the edges of the scratch wound mask in each line of resolution within an image. Wound confluence (%) represents the fractional area of the wound occupied by cells.

4.9. Statistical Analyses

Statistical significance was quantified with one-way analysis of variance (ANOVA) and Dunnett's post hoc test as performed with GraphPad Prism software (version 5, GraphPad Software, San Diego, CA, USA).

4.10. Transcriptome Analysis

HepG2, MCF-7 and MDA-MB-231 cells were plated in 6-well plates. After 24 h of serum and steroid deprivation, the cells were treated with 0.1% (*v/v*) DMSO to serve as a control, or with TCDD 1 nM, GI or GII 10 μM alone or in combination with TCDD. Total RNA was prepared and controlled for quality. Then, a high-throughput mRNA-Seq library for 3' digital gene expression (DGE) was constructed using a modified SCRBS-Seq protocol [57] with barcoded poly-dT RT primers and a hybrid Nextera/TruSeq sequencing strategy. Data quality control and primary analysis were performed by the GenoBiRD Platform in Nantes, France.

4.11. Statistical Filtration and Clustering

The data were analyzed with the Annotation, Mapping, Expression and Network (AMEN) suite of tools [58]. In summary, all probes showing a signal greater than the background cutoff (which is the overall median of the normalized dataset) and at least a 1.5-fold change over that of the control and treatment signals were selected. Then, linear models for the microarray data (LIMMA) package (F-value adjusted with the false discovery rate method, $p \leq 0.05$) [59] were used to define a set of 565 genes displaying significant statistical changes across comparisons. The resulting probes were then partitioned into nine clusters (termed C1–C9) using the k-means algorithm. The resulting clusters were ordered based on their peak expression.

4.12. Functional Analysis

A Gene Ontology enrichment analysis was performed with the AMEN suite of tools [58]. A specific annotation term was considered enriched in a gene cluster when the FDR-adjusted p value was <0.001 (Fisher's exact probability).

Supplementary Materials: Information on the Supplementary materials can be found at <http://www.mdpi.com/1422-0067/21/4/1368/s1>.

Author Contributions: T.H.P., S.L. and F.P. conceived the experiments and were the major contributors to the written original draft of the manuscript. T.H.P., S.L. and F.P. performed and analyzed the experiments. R.L.G. performed the docking analyses. F.F. conducted the animal experiments. P.B. performed the HAHLH reporter cell assays. B.E. and T.H.P. constructed the mRNA-Seq libraries for the 3' digital gene expression. A.L. and F.C. analyzed the transcriptome data. T.E. produced and provided the glyceollins. F.P. supervised and coordinated the study. All authors have read and agreed to the published version of the manuscript.

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Abbreviations

AhR	Aryl hydrocarbon receptor
GI	Glyceollin I
GII	Glyceollin II
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
BC	Breast cancer
ER	Estrogen receptor
XRE	Xenobiotic response element
EMT	Epithelial-mesenchymal transition
PAS	Per-Arnt-Sim
LBD	Ligand binding domain
HIF-2 α	hypoxia-inducible factor 2alpha

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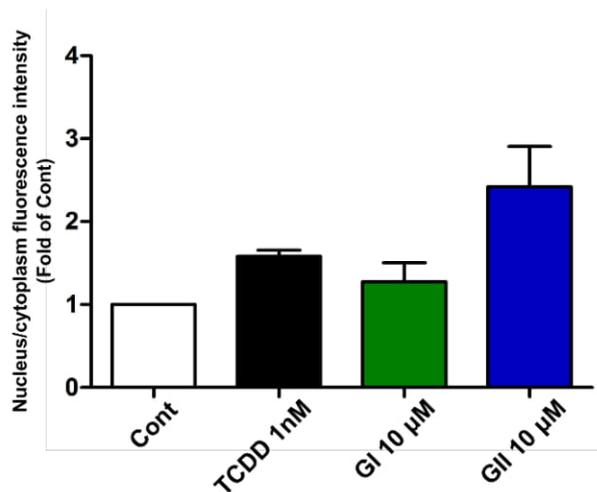
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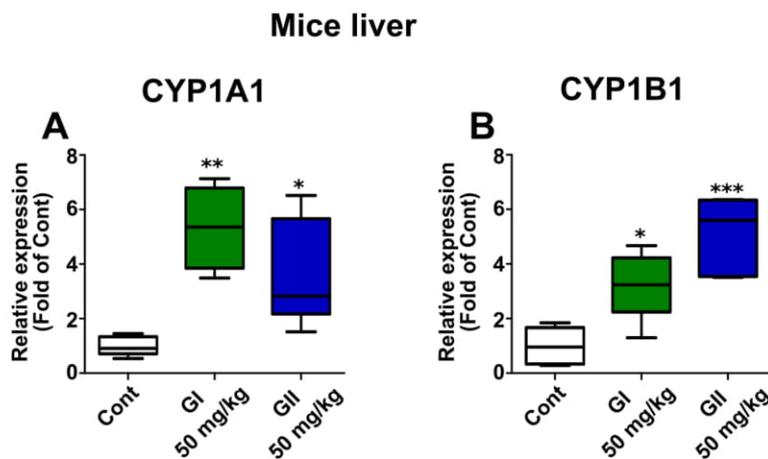
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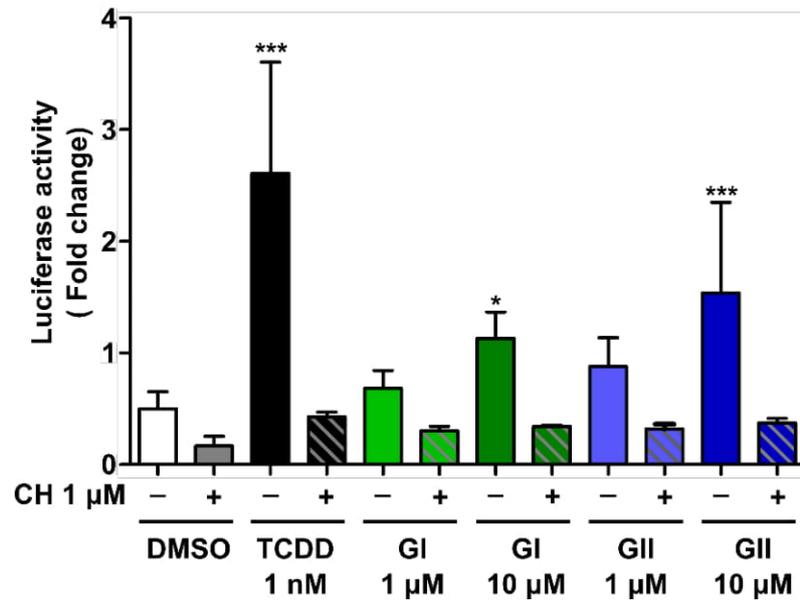
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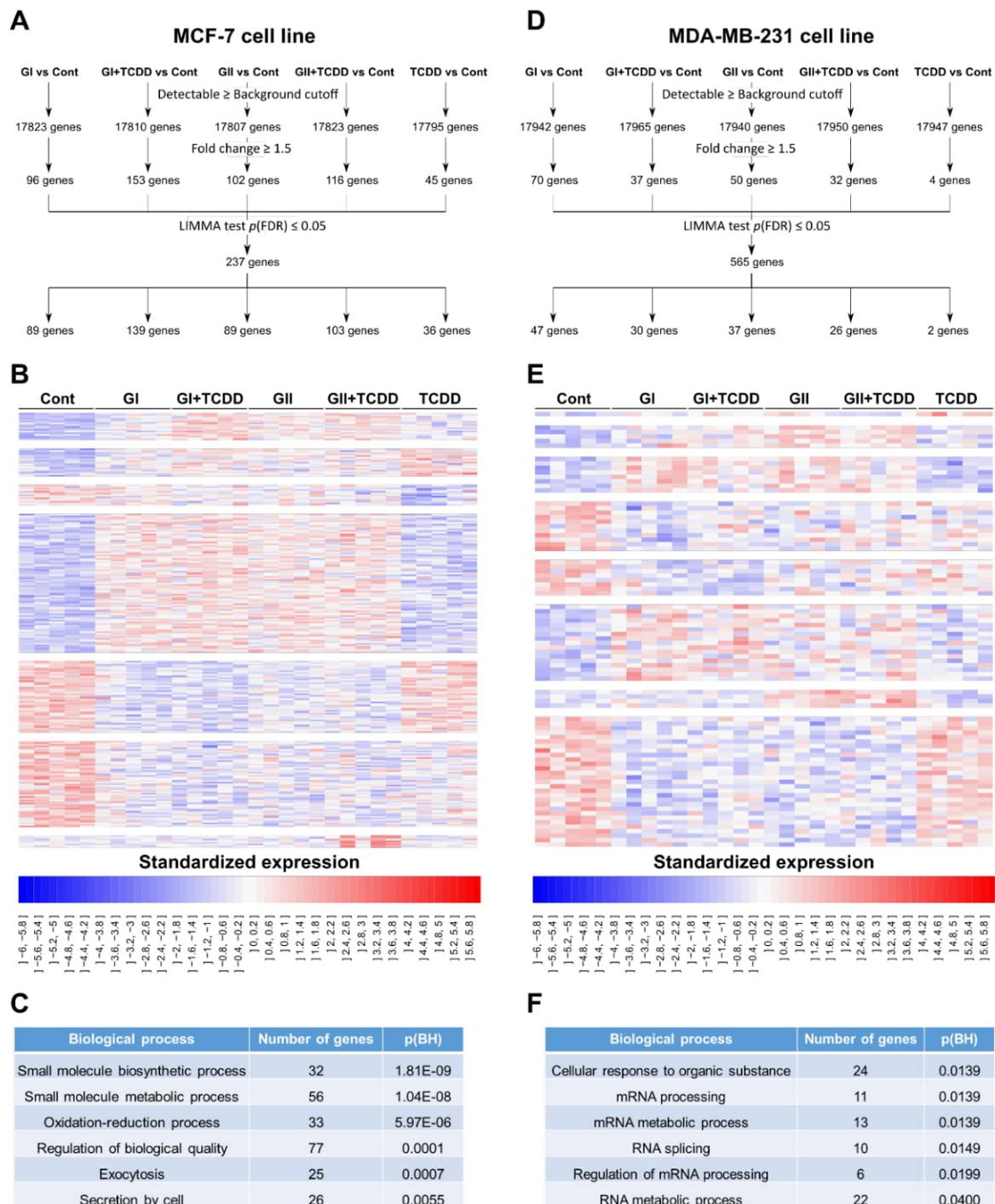
Supplementary figure 1. Nucleus/cytoplasm fluorescence intensity of AhR in HepG2 treated with DMSO at 0.1% (*v/v*) as Cont, TCDD 1nM, GI 10 µM or GII 10 µM for 24 h. Fluorescence intensity in nucleus and cytoplasm of in total 3074 cells distributed in the four treatments was measured using the software ImageJ from two independent experiments. For each experiment, nucleus/cytoplasm intensity of treated conditions is presented in fold change compared with nucleus/cytoplasm intensity of Cont condition. Data is represented as the mean ± SD.



Supplementary figure 2. Glyceollin treatment increases the level of CYP1A1 and CYP1B1 in vivo in mice liver. Mice liver came from ovariectomized RjOrl SWISS female treated by subcutaneous injection for 3 consecutive days with glyceollin I or II 50 mg/kg/day. The relative expression of CYP1A1 (A) and CYP1B1 (B) was assessed by real-time PCR and normalized to the expression of the housekeeping gene GAPDH. The results are expressed as the change fold of each group compared to the control group and are represented as the box and whiskers (10-90 percentile) of at least 4 animals per group. Statistical analyses were performed with one-way ANOVA followed by Dunnett's post hoc test. * *p*-value < 0.05, ** *p*-value < 0.01, *** *p*-value < 0.001 are considered to be significantly different with the control group.

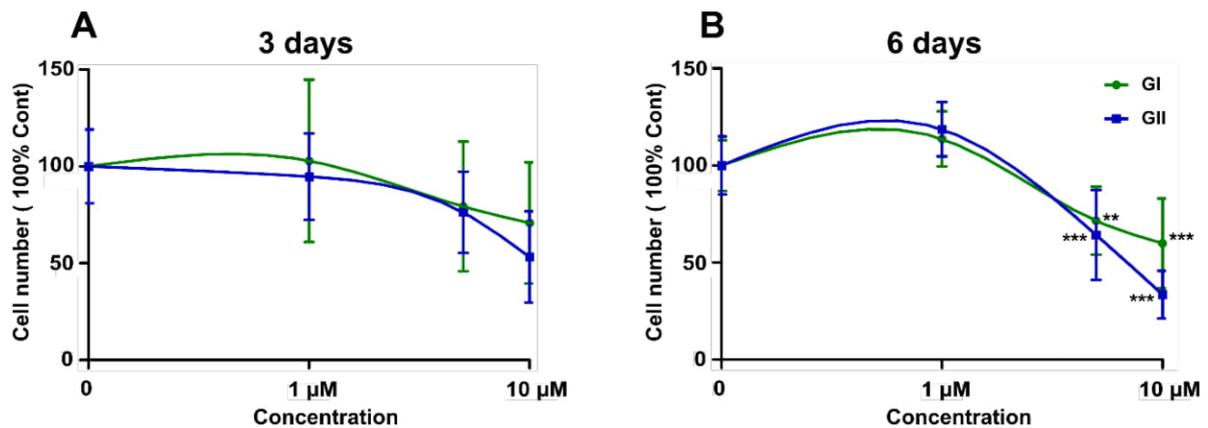


Supplementary figure 3. Glyceollins activate aryl hydrocarbon receptor (AhR) bound to XRE-containing CYP1A1. AhR-mediated transcriptional activity was analyzed by transfecting cells with an XRE-CYP1A1 promoter pHu-1A1-FL luciferase reporter plasmid and a CMV- β -galactosidase plasmid as a control for transfection efficiency. MCF-7 cells were treated for 24 h with DMSO at 0.1% (*v/v*) (white) to serve as the control (Cont), TCDD (black) at 1 nM or two different concentrations of GI (green) and GII (blue), alone or in combination with 1 μ M CH223191 (CH), an AhR antagonist. The results are expressed as the fold change in luciferase activity compared with that in the control and are represented as the mean \pm SD. The experiment was done 3 times in triplicate. Statistical analyses were performed with one-way ANOVA followed by Dunnett's post hoc test. * *p*-value < 0.05, *** *p*-value < 0.001 are considered to be significantly different with the control group.



Supplementary figure 4. Transcriptomic analysis, selection and clustering of differentially expressed genes in the MCF-7 cells and MDA-MB-231 cells. MCF-7 (A, B, C) or MDA-MB-231 (D, E, F) cells were treated with 0.1 % (v/v) DMSO to serve as the control (Cont), 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) at 1 nM, glyceollin I (GI) or glyceollin (GII) at 10 μ M alone or in combination with TCDD for 24 h. Total RNA was extracted, labeled, reverse transcribed and sequenced. (A, D) The differentially expressed genes for each treatment were selected in comparison to the control levels. Then all probes with an intensity signal greater than the overall median and a fold change ≥ 1.5 were selected. The genes were then grouped together and subjected to a LIMMA test; and only those probes with a p -value ≤ 0.05 were retained, resulting in a total of 237 differentially expressed genes for MCF-7 cells (A) and 83 differentially expressed genes for MDA-MB-231 cells (D). (B, E) 237 differentially

expressed genes for MCF-7 cells (B) and 83 differentially expressed genes for MDA-MB-231 cells (E) were clustered on the basis of their expression patterns. (C, F) Main biological processes that the differentially expressed genes by all treated conditions compares with control involve in.



Supplementary figure 5. Effect of glyceollin I and II on the proliferation of the MDA-MB-231 cell line. MDA-MB-231 cells were treated with various concentrations of glyceollin I (GI) and glyceollin II (GII). Cell numbers were determined by counting after 3 days (A) and 6 days (B) of treatment. The experiment was done 3 times in triplicate. Statistical analyses were performed with one-way ANOVA followed by Dunnett's post hoc test. ** p -value < 0.01, *** p -value < 0.001 are considered to be significantly different with the control group.

Partie 2 : Apigénine, un antagoniste partiel du récepteur des œstrogènes (ER), inhibe la prolifération des cellules cancéreuses du sein ER-positives via la signalisation Akt / FOXO3A / FOXM1

Dans le cadre du projet FUI mVolio, en plus des glycéollins, l'équipe s'est également intéressée à d'autres molécules naturelles et à leurs propriétés de SERMs. Dans une première étude de criblage, l'effet de plusieurs composés phytochimiques a été testé sur la prolifération et la différenciation cellulaire, ER-dépendante [194]. Pour cela, deux lignées cancéreuses mammaires MCF-7 et T47D ont été utilisées comme modèle de prolifération cellulaire et des cellules PC12, exprimant ER α de manière stable, ont été utilisées comme modèle de différenciation cellulaire (neuritogenèse ER-dépendante). Parmi les molécules testées, l'apigénine et le resvératrol ont montré un effet prolifératif partiel ou nul sur les cellules cancéreuses mammaires, mais, ont pleinement contribué à l'effet neuritogenèse. Inversement, la daidzéine et la zéaralénone ont montré des effets complets sur la prolifération des cellules cancéreuses mammaires, mais, n'ont pas induit la différenciation des cellules neuronales.

Une deuxième étude a permis d'éclaircir les mécanismes moléculaires pouvant expliquer l'effet différentiel de la zéaralénone et de l'apigénine [195]. Ce travail est présenté dans l'annexe. Dans cette étude, nous avons confirmé l'effet bénéfique potentiel de l'apigénine sur la santé, tandis que le zéaralénone semble présenter des caractères d'un perturbateur endocrinien. De manière intéressante, l'apigénine augmente légèrement la prolifération des cellules cancéreuses mammaires quand elle est utilisée seule mais cette molécule antagonise l'effet prolifératif de l'E2.

Les effets différentiels agoniste/antagoniste de l'apigénine sur l'activité d'ER peuvent être intéressants dans les cancers du sein, particulièrement dans les cas de l'échappement hormonal. L'objectif de la deuxième partie de cette thèse est donc d'étudier l'effet de cette molécule naturelle dans un contexte des cellules cancéreuses mammaires résistantes à l'hormonothérapie. Un des mécanismes importants de la

résistance endocrinienne est l'altération de la voie PI3K/Akt/mTOR. L'activation d'Akt, la protéine clé de cette voie, est corrélée à la résistance hormonale et prédit un résultat plus mauvais chez les patients traités par voie endocrinienne. De plus, la mutation Akt1-E17K qui contribue à la tumorigenèse mammaire a été trouvée chez 4 à 8 % des patientes atteintes d'un CS. Pour notre étude, nous avons construit des clones cellulaires MCF-7 contrôles (MCF-7/Ctrl) et activées par la voie d'Akt (MCF-7/Akt) où l'activité d'ER est modifiée. Comme attendu, les cellules MCF-7/Akt ont des niveaux plus élevés en Akt par rapport au clone MCF-7/Ctrl. L'activité d'ER ainsi que la réponse cellulaire à l'E2 sont modifiées. En ce qui concerne la signalisation Akt/FOXO3A/FOXM1, l'expression de FOXO3a est réprimée tandis que celle de FOXM1 est amplifiée dans les cellules MCF-7/Akt. De façon intéressante, l'Api renforce l'effet antiprolifératif du 4-hydroxytamoxifène (4-OHT) lorsqu'il est utilisé en combinaison. De plus, l'Api diminue l'expression de la protéine FOXM1 et de ses gènes apparentés, en particulier dans le clone MCF-7/Akt. Cette étude renforce donc le potentiel thérapeutique de l'Api dans le CS, en particulier dans le CS résistant.

**PUBLICATION N°3 « Apigenin, a Partial Antagonist
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Article

Apigenin, a Partial Antagonist of the Estrogen Receptor (ER), Inhibits ER-Positive Breast Cancer Cell Proliferation through Akt/FOXO1 Signaling

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Abstract: Approximately 80% of breast cancer (BC) cases express the estrogen receptor (ER), and 30–40% of these cases acquire resistance to endocrine therapies over time. Hyperactivation of Akt is one of the mechanisms by which endocrine resistance is acquired. Apigenin (Api), a flavone found in several plant foods, has shown beneficial effects in cancer and chronic diseases. Here, we studied the therapeutic potential of Api in the treatment of ER-positive, endocrine therapy-resistant BC. To achieve this objective, we stably overexpressed the constitutively active form of the Akt protein in MCF-7 cells (named the MCF-7/Akt clone). The proliferation of MCF-7/Akt cells is partially independent of estradiol (E2) and exhibits an incomplete response to the anti-estrogen agent 4-hydroxytamoxifen, demonstrating the resistance of these cells to hormone therapy. Api exerts an antiproliferative effect on the MCF-7/Akt clone. Api inhibits the proliferative effect of E2 by inducing G2/M phase cell cycle arrest and apoptosis. Importantly, Api inhibits the Akt/FOXO1 signaling pathway by decreasing the expression of FOXO1, a key transcription factor involved in the cell cycle. Api also alters the expression of genes regulated by FOXO1, including cell cycle-related genes, particularly in the MCF-7/Akt clone. Together, our results strengthen the therapeutic potential of Api for the treatment of endocrine-resistant BC.

Keywords: breast cancer; endocrine resistance; ER; Akt; FOXO1; phytochemicals; apigenin



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1. Introduction

Breast cancer (BC) is the most commonly diagnosed cancer and the leading cause of cancer-related death in women worldwide; nearly 2.1 million new cases were diagnosed in 2018 [1]. Among the different types of BC, estrogen receptor (ER)-positive subtypes, namely, Luminal A and Luminal B, represent approximately 80% of cases [2]. These subtypes tend to be less immediately aggressive than other subtypes and can initially respond to endocrine treatment [2]. However, 15 to 20% of these tumors are intrinsically resistant to treatment, and 30 to 40% acquire resistance to treatment over a period of several years [3]. Approximately 40% of patients with BC acquire endocrine resistance following therapy with tamoxifen [4]. One of the important mechanisms by which endocrine resistance is acquired is alterations in the PI3K/Akt/mTOR pathway [5,6]. The activation of Akt, the key protein in this pathway, is correlated with hormone resistance and predicts a worse outcome in endocrine therapy-treated patients [7,8]. The Akt1-E17K mutation has been observed in 4 to 8% of BC patients, can lead to Akt activation and contribute to mammary tumorigenesis [9]. Therefore, Akt is an important therapeutic target, in order to identify new treatments for endocrine-resistant BC.

The PI3K/Akt signaling pathway is one of the upstream kinase pathways that regulate Forkhead box protein M1 (FOXO1), which is an essential proliferation-associated transcription factor that regulates the cell cycle [10,11]. FOXO1 regulates all the hallmarks of cancer,

including proliferation, mitosis, EMT, invasion, and metastasis [10]. In ER-positive BC, increased expression of FOXM1 is associated with increased cell invasiveness and resistance to endocrine treatments. Consequently, increased FOXM1 expression is correlated with significantly reduced survival in BC patients [12]. Akt can promote the activity of FOXM1 by blocking the activity of FOXO3a, which antagonizes the activity of FOXM1 [13]. Therefore, the FOXO3a-FOXM1 axis is actually a crucial therapeutic target for the treatment of cancer, particularly for overcoming drug resistance [14].

BC is a multifactorial disease, and certain known factors, including diet, can increase or decrease the risk of contracting the disease [15–17]. Epidemiological studies and systematic analyses suggest that diets rich in flavonoids are inversely associated with a risk of BC [18–21]. Among these flavonoids, apigenin (4',5,7-trihydroxyflavone) (Api) is one of the most frequently studied phenolic molecules because it is very widely expressed in the plant kingdom and it is common in teas, dry herbs, fruits, and vegetables [22]. Api is abundantly present in celery, parsley, and dried chamomile flowers [22] as well as in herbs that have been used in traditional Chinese medicine for centuries [23]. Recent studies have shown that Api can inhibit the growth of BC cells and xenograft tumors both, in hormone receptor-positive BC [24–27] and in triple-negative breast cancer (TNBC) [28–31]. In hormone receptor-positive BC, Api can block the expression of mucin 1 C-terminal subunit oncoprotein [24] and induce apoptosis by activating p53 and inhibiting STAT3 and NFκB [26]. In TNBC, Api suppresses stem cell-like properties by inhibiting YAP/TAZ activity [30] and blocks IL-6 expression [31]. Moreover, Api can inhibit the mechanisms of drug resistance and enhance the efficiency of chemotherapy [32–35]. Therefore, Api may be a potential treatment or adjuvant for endocrine-resistant BC.

In the current study, we investigated the effect of Api in ER-positive MCF-7 (MCF-7/Ctrl) BC cells and Akt-activated MCF-7 (MCF-7/Akt) BC cells. We observed that in addition to the antagonistic effect of Api on E2-induced proliferation, treatment with Api alone decreased MCF-7/Akt cell proliferation. This effect of Api enhanced the effect of 4-hydroxytamoxifen (4-OHT) when the two agents were used in combination. Regarding ER signaling, Api exerted an agonist/antagonist effect on ER transcriptional activity and on ER target gene expression. Regarding Akt/FOXO3a/FOXM1 signaling, the expression of FOXO3a was suppressed and the expression of FOXM1 was enhanced in MCF-7/Akt cells compared with MCF-7/Ctrl cells. Api treatment decreased the protein expression of FOXM1 and its related genes, especially in the MCF-7/Akt clone. Together, our results support the therapeutic potential of Api in BC, particularly in endocrine-resistant BC.

2. Results

2.1. Construction and Characterization of Akt-Activated MCF-7 (MCF-7/Akt) Cells and MCF-7/Ctrl Cells

As described in the Methods section, we constructed MCF-7 cells in which Akt was constitutively active (MCF-7/Akt cells) by transfecting parent MCF-7 cells with the myr-Akt1 cDNA vector using the T-Rex™ system. The myr-Akt1 construct contains the 11 N-terminal amino acids of the avian sequence fused in frame to the N-terminus of the wild-type Akt coding sequence. These additional amino acids, which are required for protein myristoylation, allow the attachment of Akt to the intracellular side of the cell membrane. Therefore, Akt can be activated without the activation of its upstream proteins. Western blot analyses using an anti-Akt antibody revealed that the MCF-7/Akt cells treated with tetracycline strongly expressed constitutively active Akt, which has a larger molecular mass than wild-type Akt due to the 11 additional amino acids (Figure 1A).

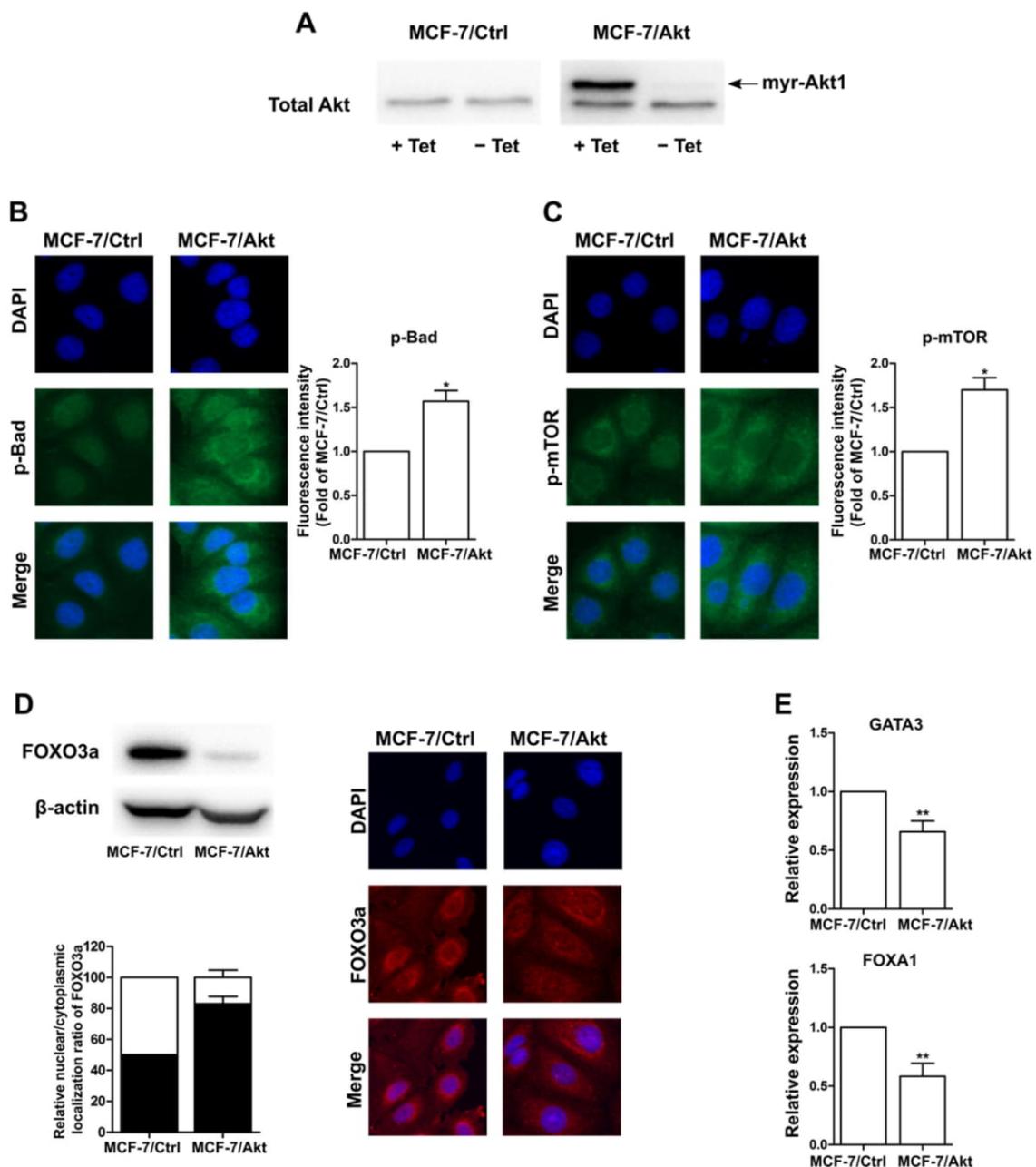


Figure 1. Characterization of MCF-7 cell clones expressing wild-type Akt (MCF-7/Ctrl) and its constitutively active form (MCF-7/Akt). (A) Equal amounts of protein extracts from MCF-7/Ctrl cells and MCF-7/Akt cells treated with or without tetracycline (Tet) were analyzed by Western blotting with an antibody specific for Akt. Notably, myr-Akt1 has a larger molecular mass than wild-type Akt1. (B,C) Protein expression and subcellular localization of phospho-Bad (B) and phospho-mTOR (C) in MCF-7/Ctrl cells and MCF-7/Akt cells, as examined by immunofluorescence. (D) Equal amounts of protein extracts from MCF-7/Ctrl cells and MCF-7/Akt cells were analyzed by Western blotting with an antibody specific for FOXO3a (left, above). Protein expression and subcellular localization of FOXO3a in MCF-7/Ctrl cells and MCF-7/Akt cells examined by immunofluorescence (right). Quantification of the nuclear/cytoplasmic localization ratio of FOXO3a (left, below). Fluorescence intensity in the nucleus and cytoplasm of each cell was measured using ImageJ software. The median nuclear/cytoplasmic localization ratio of the control condition was used as a threshold to determine the percentage of cells below (black) and above (white) this threshold in each condition. (E) Gene expression of GATA3 (above) FOXA1 and (below) in MCF-7/Ctrl cells and MCF-7/Akt cells, as revealed by RT-PCR. Statistical analyses were performed with a Mann-Whitney test. * p -value < 0.05, ** p -value < 0.01 is considered to be significantly different from the MCF-7/Ctrl cell group.

Next, we examined the expression and subcellular localization of the two proteins downstream of Akt, namely, phospho-Bad and phospho-mTOR, using immunofluorescence. We observed an increase in the phospho-Bad and phospho-mTOR levels in the MCF-7/Akt cells compared with the MCF-7/Ctrl cells (Figure 1B,C). Moreover, the expression of these proteins was almost increased in the cytoplasm. On the other hand, it is well documented that the Akt-mediated phosphorylation of FOXO proteins induces their cytoplasmic translocation and degradation [13]. Thus, we evaluated the protein level and nuclear/cytoplasmic localization of FOXO3a in the two MCF-7 clones. As shown by the Western blot analyses in Figure 1D, we observed a sharp decline in the protein expression of FOXO3a in the MCF-7/Akt cells. Moreover, immunofluorescence analyses showed a decrease in the ratio of nuclear/cytoplasmic localization of FOXO3a in the MCF-7/Akt cells compared to the MCF-7/Ctrl cells (Figure 1D). Furthermore, we observed a decrease in the gene expression of two pioneer transcription factors, GATA3 and FOXA1, in the MCF-7/Akt cells (Figure 1E). Since these factors can associate with compacted chromatin to facilitate the binding of additional transcription factors, including ER [36], the decrease in these factors in the MCF-7/Akt cells may alter ER activity and enhance cell transformation.

Overall, we confirmed the constitutive activation of Akt in MCF-7/Akt cells. The results also show that activation of Akt negatively regulates FOXO3a by significantly decreasing its protein levels and increasing its exclusion from the nucleus and its presence in the cytoplasm.

2.2. Effects of Api on the Proliferation of MCF-7/Ctrl Cells and MCF-7/Akt Cells

To determine the effect of Api on cell proliferation, MCF-7/Ctrl cells and MCF-7/Akt cells were cultured in the presence or absence of Api alone or Api with E2 (Figure 2A). Compared with the MCF-7/Ctrl cells, which proliferate very slowly in the absence of E2, the MCF-7/Akt cells grew independently of E2. Then, the proliferation of the MCF-7/Ctrl cells increased by nearly four-fold with E2 treatment, while the proliferation of the MCF-7/Akt cells increased by less than two-fold. Compared with solvent alone, Api alone increased the proliferation of the MCF-7/Ctrl cells, but interestingly, it decreased the proliferation of the MCF-7/Akt cells. On the other hand, the combination of Api and E2 decreased the E2-induced proliferation of both the MCF-7/Ctrl cells and MCF-7/Akt cells.

Next, we compared the effect of 4-hydroxytamoxifen (4-OHT) alone or 4-OHT in combination with Api, in the presence or absence of E2, in the two clones (Figure 2B). We observed that in the MCF-7/Ctrl cells, 4-OHT alone slightly but significantly inhibited proliferation compared with solvent alone. Moreover, 4-OHT antagonized the effect of E2 on cell proliferation. The effect of 4-OHT was not altered when the MCF-7/Ctrl cells were also treated with Api. However, the baseline proliferation of the MCF-7/Akt cells was not affected by 4-OHT treatment. However, the combination of 4-OHT and Api enhanced the antiproliferative effect of 4-OHT in this clone. Together, our results confirm that the constitutive activation of Akt in MCF-7 cells induces the emergence of hormone resistance that is characterized by autonomous growth and the acquisition of characteristics that render MCF-7 cells resistant to 4-OHT. Api partially reduces this resistance by enhancing the antiproliferative effect of 4-OHT.

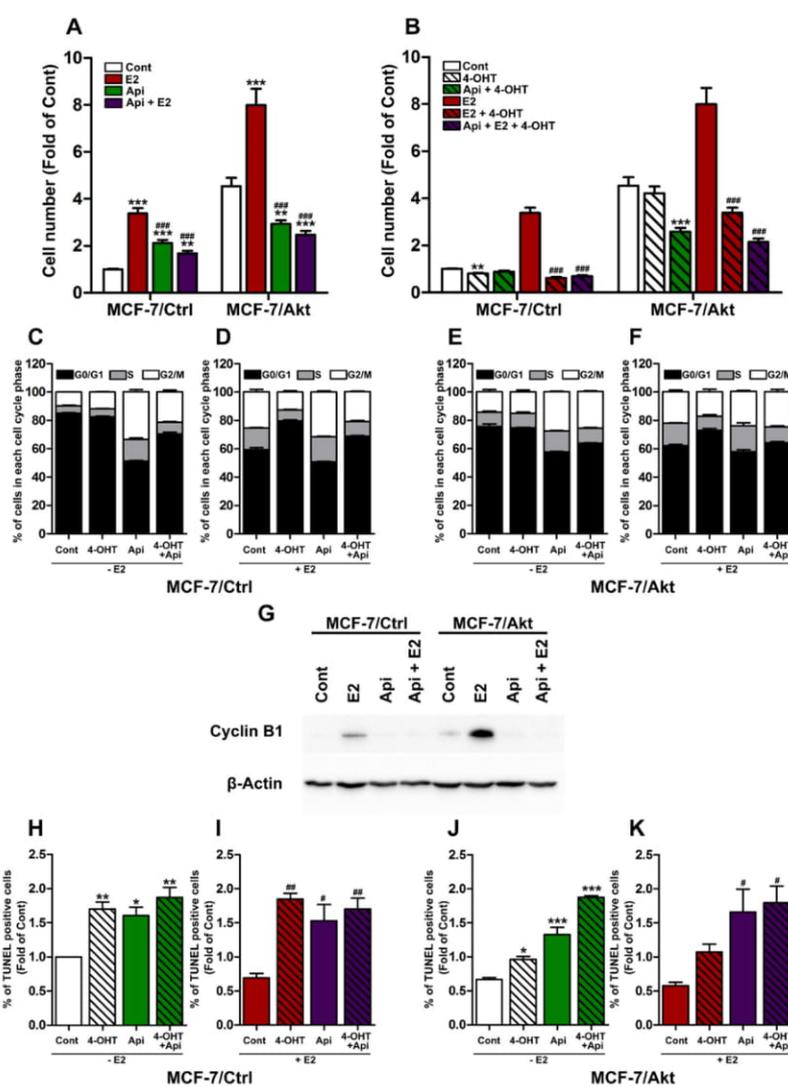


Figure 2. Effects of apigenin on the proliferation and cell cycle of MCF-7/Ctrl cells and MCF-7/Akt cells. (A) MCF-7/Ctrl cells and MCF-7/Akt cells were treated with solvent (0.1% (*v/v*) DMSO and 0.1% (*v/v*) ethanol) as the control (Cont), 1 nM estradiol (E2), or 10 μ M apigenin (Api) alone or in combination with E2. (B) MCF-7/Ctrl cells and MCF-7/Akt cells were treated with solvent (Cont) or 1 μ M 4-hydroxytamoxifen (4-OHT) alone or in combination with Api in the presence or absence of E2. Cell numbers were determined using the sulforhodamine B assay after 6 days of treatment. The experiment was conducted 3 times in quintuplicate. The results are expressed as the fold change in cell number compared with that in the control MCF-7/Ctrl cells and are presented as the mean \pm SEM. For the cell cycle assays, MCF-7/Ctrl cells (C,D) and MCF-7/Akt cells (E,F) were treated with solvent (Cont), 4-OHT and Api alone or in combination in the presence or absence of E2 for approximately 24 h. The cell cycle was analyzed by flow cytometry after propidium iodide staining. The experiment was conducted 3 times. The results are expressed as the percentage of cells in each phase of the cell cycle and are presented as the mean \pm SEM. (G) Equal amounts of protein extracts from MCF-7/Ctrl cells and MCF-7/Akt cells were analyzed by Western blotting with antibodies specific for cyclin B1 and β -actin. MCF-7/Ctrl cells and MCF-7/Akt cells were treated with solvent (Cont), E2 and Api alone or in combination with E2 for 24 h. For apoptosis analysis, MCF-7/Ctrl cells (H,I) and MCF-7/Akt cells (J,K) were treated with solvent (Cont), 4-OHT and Api alone, or in combination, in the presence or absence of E2 for approximately 24 h. Apoptotic cells were detected using terminal deoxynucleotidyl dUTP nick end labeling (TUNEL) assays. The experiment was conducted 3 times. The percentage of apoptotic cells was quantified and expressed as the fold change of the Cont-treated of the MCF-7/Ctrl clone. The results are presented as the mean \pm SEM. Statistical analyses were performed with one-way ANOVA followed by Tukey's post hoc test. * *p*-value < 0.05, ** *p*-value < 0.01, *** *p*-value < 0.001 indicate significant differences from the control group in the same clone. # *p*-value < 0.05, ## *p*-value < 0.01, ### *p*-value < 0.001 indicate significant differences from the E2 group in the same clone.

Since the cell cycle plays an important role in cell proliferation, we assessed the effects of all the treatments on the cell cycle in both MCF-7 clones. The percentage of cells in each phase of the cell cycle was determined by flow cytometry after propidium iodide staining. In the MCF-7/Ctrl cells, approximately 85% of the cells were observed to be in the G0/G1 phase, 5% in the S phase, and 10% in the G2/M phase (Figure 2C). However, in the MCF-7/Akt cells, the percentage of cells in the S phase was two times higher, and 15% of the cells were observed to be in the G2/M phase (Figure 2E). As expected, E2 treatment increased the proportion of cells in the S and G2/M phases in the two MCF-7 clones, and the fold change was greater in the MCF-7/Ctrl cells than in the MCF-7/Akt cells (Figure 2D,F). Surprisingly, Api treatment also exerted agonistic effects similar to those observed with E2 treatment by increasing the percentage of cells in the S and G2/M phases in both clones, although overall, the increase in the cells in the G2/M phases induced by Api was more prominent than that induced by E2 (Figure 2C–F). The 4-OHT treatment decreased the percentage of cells in the S and G2/M phases and antagonized the effects of E2 and Api on the cell cycle when used in combination with these treatments (Figure 2C–F).

To further distinguish the effect of Api from that of E2 on the cells in the G2/M phases, we examined the effects of these treatments on the expression of a marker of these cell phases, namely, cyclin B1, which is an essential protein for the G2/M phases (Figure 2G). While E2 clearly increased the expression of this protein in the two clones, Api treatment alone did not change the expression of cyclin B1 in the MCF-7/Ctrl cells and decreased its expression in the MCF-7/Akt cells. When the cells were treated with Api in combination with E2, Api blocked the effects of E2. Since cyclin B1 plays an essential role in the G2/M phase transition by enhancing mitochondrial function in order to provide energy for this transition [37], deficiency of cyclin B1 under Api treatment may cause a reduction in the number of cells in the M phase. These results could partially explain why both E2 and Api increase the number of cells in the S and G2/M phases, but E2 exerts proliferative effects and Api exerts anti-proliferative effects.

Since the cell number is the result of cell growth and cell death, we next analyzed the effects of Api on apoptosis (Figure 2H–K). We observed that the baseline percentage of apoptotic cells in the MCF-7/Ctrl (Figure 2H) was higher than that in the MCF-7/Akt cells (Figure 2J). As expected, E2 treatment decreased the percentage of apoptotic cells in the MCF-7/Ctrl, while the effect of E2 was much weaker in the MCF-7/Akt cells. More importantly, treatment with 4-OHT or Api increased the percentage of apoptotic cells in the two cell models in the presence or absence of E2 (Figure 2H–K). The combination of 4-OHT and Api in absence of E2 had a synergistic effect on the MCF-7/Akt cells (Figure 2J), which was consistent with the results of the cell counts (Figure 2B).

Together, our results confirmed the anti-proliferative effects of Api in E2-dependent MCF-7/Ctrl cells and in partially E2-independent MCF-7/Akt cells. These effects are due to the Api-induced G2/M phase arrest and apoptosis.

2.3. Effects of Apigenin on ER Genomic Activity

To better understand the molecular mechanisms involved in the anti-proliferative effect of Api on the two clones, we first investigated the effects of Api on ER signaling. At the gene expression level, ER α expression was increased in the MCF-7/Akt clone compared to the MCF-7/Ctrl clone. E2 treatment decreased ER α gene expression in the two clones, and cotreatment with Api completely attenuated the effect of E2 in the MCF-7/Ctrl cells, but the effect was not significant in the MCF-7/Akt cells (Figure 3A). In relation to the effects of Api on ER target gene expression, Api exerted an agonist/antagonist effect on the gene expression of PgR (Figure 3B). However, Api exerted a complete agonist effect on the gene expression of AREG (Figure 3C) and CXCL12 (Figure 3D). Comparing the two clones, the baseline level of PgR increased in the MCF-7/Akt cells compared to the MCF-7/Ctrl cells. Notably, the baseline level of AREG dramatically decreased in the MCF-7/Akt cells.

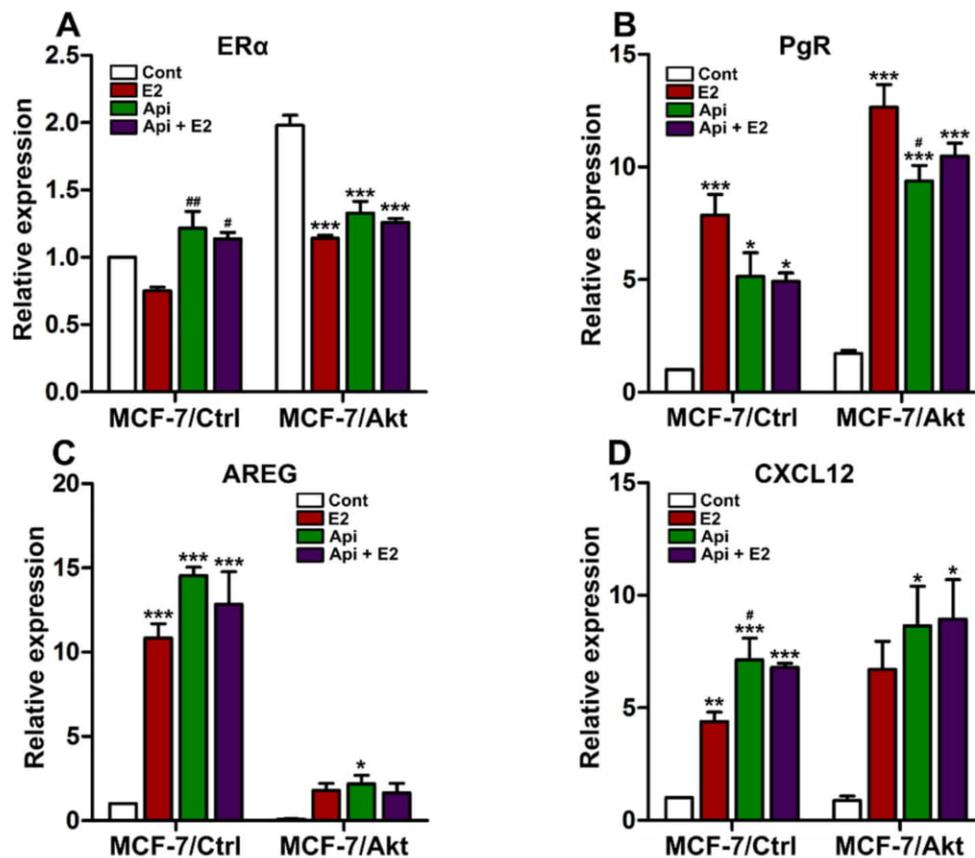


Figure 3. Effect of apigenin on ER target genes. MCF-7/Ctrl cells and MCF-7/Akt cells were treated with 0.1% (*v/v*) DMSO and 0.1% (*v/v*) ethanol as the control (Cont), 1 nM estradiol (E2), or 10 μ M apigenin (Api) alone or in combination with E2 for 24 h. The gene expression of estrogen receptor 1 (ER α) (A), progesterone receptor (PgR) (B), amphiregulin (AREG) (C) and chemokine (C-X-C motif) ligand 12 (CXCL12) (D) was assessed by real-time PCR and normalized to the expression of the housekeeping genes GAPDH and TBP. The experiment was conducted three times in triplicate. The results are expressed as the fold change in gene expression compared with that in the control of the MCF-7/Ctrl cells and are presented as the mean \pm SEM. Statistical analyses were performed with one-way ANOVA followed by Tukey's post hoc test. * *p*-value < 0.05, ** *p*-value < 0.01, *** *p*-value < 0.001 indicate significant differences from the control group in the same clone. # *p*-value < 0.05, ## *p*-value < 0.01 indicate significant differences from the E2 group in the same clone.

To further understand why Api has agonist/antagonist effects on the expression of ER-dependent genes but complete agonist effects on other ER-dependent genes, we investigated the effects of Api on ER activation via different reporter genes using luciferase assays. Api exerted complete agonist effects on the estrogen-responsive element (ERE)-luciferase reporter gene in the absence and presence of E2 (Figure 4A). However, Api exerted an agonistic effect when used alone, but an antagonistic effect when used in combination with E2 on the activation of activator protein 1 (AP1)- and specificity protein 1 (SP1)-luciferase reporter genes (Figure 4B,C). These results indicate that the antagonistic effect of Api on the ER pathway when combined with E2 could be related to the action of ER on the AP1 and SP1 pathways, but not the ERE. Of note, a similar pattern of luciferase activity was also observed in both clones.

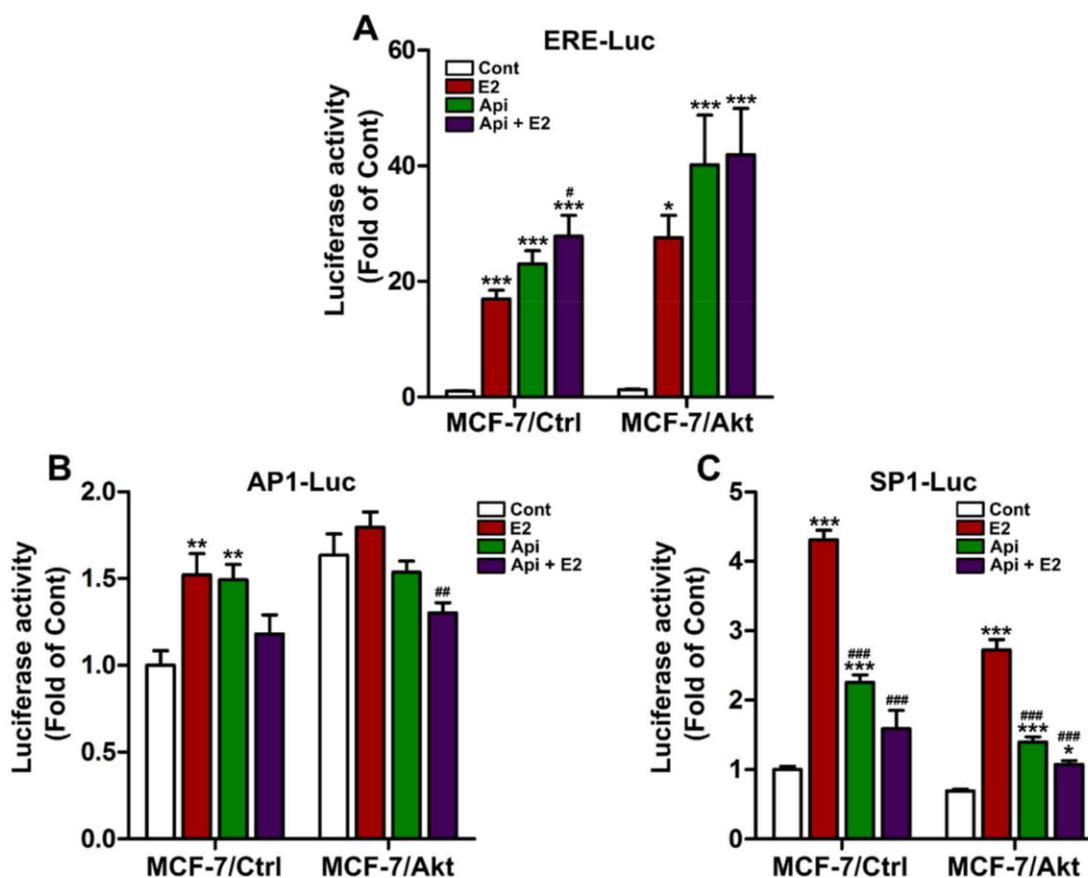


Figure 4. Effects of apigenin on the ER genomic activity. MCF-7/Ctrl cells and MCF-7/Akt cells were transiently transfected with an estrogen-responsive element luciferase reporter plasmid (ERE-Luc) (A), activator protein 1 luciferase reporter plasmid (AP1-Luc) (B) or specificity protein 1 luciferase reporter plasmid (SP1-Luc) (C) as well as a CMV- β -galactosidase plasmid as a control to assess transfection efficiency. Then, the cells were treated with 0.1% (*v/v*) DMSO and 0.1% (*v/v*) ethanol as the control (Cont), 1 nM estradiol (E2), or 10 μ M apigenin (Api) alone or in combination with E2 for 24 h. The experiment was conducted 3 times in triplicate. The results are expressed as the fold change in luciferase activity compared with that in the control of the MCF-7/Ctrl cells and are presented as the mean \pm SEM. Statistical analyses were performed with one-way ANOVA followed by Tukey's post hoc test. * *p*-value < 0.05, ** *p*-value < 0.01, *** *p*-value < 0.001 indicate significant differences from the control group in the same clone. # *p*-value < 0.05, ## *p*-value < 0.01, ### *p*-value < 0.001 indicate significant differences from the E2 group in the same clone.

2.4. Effects of Apigenin on the Expression of FOXM1 and FOXM1-Related Genes

It has been well-demonstrated that FOXM1, a transcriptional regulator of G1/S progression, is also crucial for G2/M transition [38,39]. In addition, FOXO3a is a negative regulator of the expression and activity of FOXM1. In fact, FOXO3a behaves as a tumor suppressor, while FOXM1 is a potent oncogene that is widely overexpressed in endocrine-resistant cancers [13,14]. Since FOXO3a protein expression and nuclear localization were altered in the MCF-7/Akt cells, we examined the effect of Api on FOXM1. As demonstrated by Western blot analyses, the FOXM1 protein level was clearly increased in the MCF-7/Akt cells compared to the MCF-7/Ctrl cells (Figure 5A). E2 treatment strongly increased the FOXM1 protein expression level in both the MCF-7 cell clones, and interestingly, cotreatment with Api markedly reduced the effect of E2 (Figure 5A). Notably, compared to the control treatment, Api treatment alone decreased the FOXM1 protein level in the MCF-7/Akt cells, in which the baseline expression of FOXM1 was much higher than that in the MCF-7/Ctrl cells. Moreover, E2 treatment enhanced the translocation of FOXM1

from the cytoplasm to the nucleus, while cotreatment with Api reversed the effect of E2 (Supplementary Figure S2).

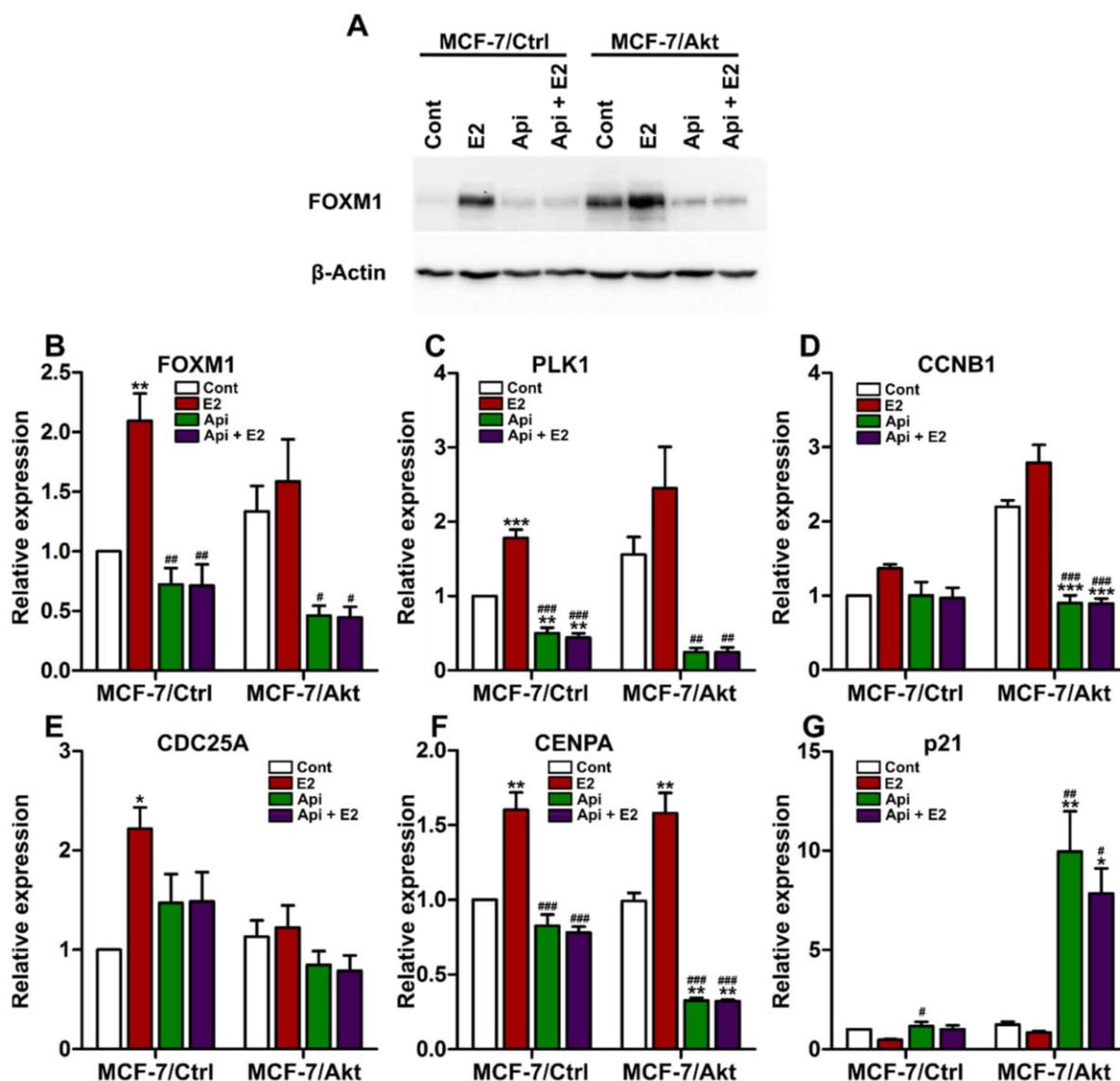


Figure 5. Apigenin inhibits the expression of FOXM1 and modulates the expression of FOXM1-related cell cycle genes. MCF-7/Ctrl cells and MCF-7/Akt cells were treated with 0.1% (*v/v*) DMSO and 0.1% (*v/v*) ethanol as the control (Cont), 1 nM estradiol (E2), or 10 μ M apigenin (Api) alone or in combination with E2 for 24 h. (A) Equal amounts of protein extracts from MCF-7/Ctrl cells and MCF-7/Akt cells were analyzed by Western blotting with antibodies specific to FOXM1 and β -actin. The gene expression of forkhead box M1 (FOXM1) (B), polo-like kinase 1 (PLK1) (C), cyclin B1 (CCNB1) (D), cell division cycle 25A (CDC25A) (E), centromere protein A (CENPA) (F) and cyclin-dependent kinase inhibitor 1A (CDKN1A/p21) (G) was assessed by real-time PCR and normalized to the expression of the housekeeping genes GAPDH and TBP. The experiment was conducted three times in triplicate. The results are expressed as the fold change in gene expression compared with that in the control of the MCF-7/Ctrl cells and are presented as the mean \pm SEM. Statistical analyses were performed with one-way ANOVA followed by Tukey's post hoc test. * *p*-value < 0.05, ** *p*-value < 0.01, *** *p*-value < 0.001 indicate significant differences from the control group in the same clone. # *p*-value < 0.05, ## *p*-value < 0.01, ### *p*-value < 0.001 indicate significant differences from the E2 group in the same clone.

Similar to the protein expression, the baseline gene expression of FOXM1 was higher in the MCF-7/Akt cells than in the MCF-7/Ctrl cells (Figure 5B). Compared with the control, E2 treatment increased the expression of FOXM1 in the MCF-7/Ctrl cells, but this effect was not significant in the MCF-7/Akt cells. More importantly, Api treatment decreased the expression of FOXM1 when administered alone and antagonized the effect of E2 when administered in combination with E2. Notably, the inhibitory effect of Api on FOXM1 gene expression in the MCF-7/Akt cells was stronger than that in the MCF-7/Ctrl cells.

The cell cycle checkpoints, especially at the G1/S and G2/M phases, are mediated by FOXM1 via the regulation of key cell cycle-associated genes, such as polo-like kinase 1 (PLK1), cyclin B1 (CCNB1), cell division cycle 25A (CDC25A), centromere protein A (CENPA), and cyclin-dependent kinase inhibitor 1A (CDKN1A, also called p21). These genes are known to be transcriptional targets of FOXM1. A similar gene expression profile to that of FOXM1 was found for PLK1 (Figure 5C), CCNB1 (Figure 5D), CDC25A (Figure 5E), and CENPA (Figure 5F). E2 treatment increased the expression of these genes, and cotreatment with Api antagonized the effects of E2. As observed for FOXM1 expression, the inhibitory effect of Api on the expression of the PLK1 and CCNB1 genes was stronger in the MCF-7/Akt cells than in the MCF-7/Ctrl cells. The results observed for the mRNA expression of CCNB1 were similar to those observed for the protein expression of CCNB1 presented in Figure 2G. Finally, as shown in Figure 5G, treatment with E2 reduced the expression of p21 by approximately two-fold. Conversely, treatment with Api increased p21 expression, and this effect was markedly observed in the MCF-7/Akt cells.

Overall, our results show that apigenin alone or in combination with E2 inhibits the expression of FOXM1, which plays an important role in the antiproliferative effects of Api in MCF-7/Akt cells.

3. Discussion

Targeted cancer therapies are drugs that inhibit specific molecular targets that are responsible for enhanced tumor growth [40]. In BC, the hyperactivation of Akt is associated with resistance to endocrine therapies [7,8]. However, to date, no drugs that target Akt have been approved for clinical use due to their limited efficiency or toxicity [40]. Targeting signaling pathways whose activities are stimulated in BC tumors with overactivated Akt is one of the strategies for combatting this disease. In this study, we focused on FOXO3a/FOXM1 signaling, which is regulated by Akt [14].

Api, an edible plant-derived flavonoid, has attracted attention because of its anticancer effects, as shown in several experimental and biological studies [41]. Api leads to cell growth arrest and apoptosis in different tumor types by modulating several signaling pathways [41]. As a potential treatment or adjuvant for endocrine-resistant BC [32–34], more studies are needed to elucidate the mechanisms of action of Api. To the best of our knowledge, our study is the first to investigate the effect of Api in breast cancer cells with activated Akt.

In this study, we first established MCF-7 cells by which Akt was constitutively active. This approach allowed us to compare the effect of Api on the proliferation of these cells and that of the parental MCF-7/Ctrl cells. As we have previously reported in different ER-positive BC cells [42], Api also exerted a partial antagonistic effect on MCF-7/Ctrl cell proliferation. However, Api exerted a completely antagonistic effect in MCF-7/Akt breast cancer cells. This discrepancy may be explained by the action of Api in signaling pathways that are strongly activated and can promote the proliferation of MCF-7/Akt cells.

Furthermore, we reported that MCF-7/Akt cells were resistant to 4-OHT treatment and that Api partially reduced this resistance by enhancing the anti-proliferative effect of 4-OHT. These observations are consistent with other studies that have shown that Api can inhibit drug resistance mechanisms and improve efficacy [32–35]. Liu et al. showed that Api increases cytotoxic effects of cisplatin through p53-modulated apoptosis [32]. In adriamycin-resistant breast cancer cells, Api overcomes drug resistance by blocking the signal transducer and activator of transcription 3 [33]. Moreover, Api exerts inhibitory

effects on breast cancer resistance protein (BCRP) and decreases the BCRP-mediated efflux of doxorubicin and temozolomide [34]. Recently, Sudhakaran et al. showed that Api sensitizes triple-negative BC spheroids to doxorubicin by targeting hnRNPA2 [35]. Notably, our study is the first to investigate the combination of 4-OHT and Api in the context of BC cells with activated Akt.

The next step of our study was to better understand the mechanisms involved in the effects of Api on breast cancer cell proliferation. First, with respect to ER signaling, Api exerted agonist/antagonist effects on some ER target genes, but complete agonist effects on others. This observation can be explained by the fact that Api exerted agonist/antagonist effects on the activation of ER signaling via AP1 and SP1 but exerted complete agonist effects via the ERE. These effects on ER signaling can explain the agonist/antagonist effects of Api on MCF-7/Ctrl cell proliferation and the antagonist effect of Api when administered with E2 in MCF-7/Akt cells. However, the effects of Api on ER activity cannot explain the antiproliferative effects of treatment with Api alone on the MCF-7/Akt clone. The anti-proliferative effects of Api on MCF-7/Akt cells may act through other signaling pathways.

One explanation for the resistance of our MCF-7/Akt cells to 4-OHT treatment could be the increased expression of FOXM1. The correlation between elevated FOXM1 expression and endocrine resistance in breast tumors has previously been well-demonstrated [12]. Indeed, the FOXM1 oncogene is positively regulated by both ER and PI3K/Akt/FOXO3a signaling [14]. Akt negatively regulates the FOXO3a protein by inducing its exclusion from the nucleus and presence in the cytoplasm, where FOXO3a undergoes degradation [14]. Consistently, in the MCF-7/Akt cells, in which Akt was constitutively activated, we observed a drastic decrease in the FOXO3a protein levels and in FOXO3a translocation from the nucleus to the cytoplasm. We also observed that the FOXO1 proteins levels are clearly lower in the MCF-7/Akt cells than in the MCF-7/Ctrl cells (Supplementary Figure S3). Consequently, the level of FOXM1, which is negatively regulated by the FOXOs proteins, was clearly increased in the MCF-7/Akt cells compared to the MCF-7/Ctrl cells.

We observed no clear effects of Api on the expression or nuclear/cytoplasmic localization of FOXO3a (data not shown). Regarding the FOXO1 protein, there is a slight but not significant increase of its expression under Api treatment in MCF-7/Akt cells (Supplementary Figure S3). However, it is interesting to note that Api acts at the levels of both FOXM1 expression and activity. Api treatment decreased FOXM1 expression at both the gene and protein levels in the MCF-7/Akt cells, and the level of FOXM1 was higher in the MCF-7/Akt cells than in the MCF-7/Ctrl cells. Moreover, Api treatment also decreased the E2-induced stimulation of FOXM1 expression in the two clones. A similar profile was found in FOXM1-related genes involved in the cell cycle. In particular, the inhibitory effects of Api on cyclin B1, which is a protein that is essential for the G2/M transition, can explain the anti-proliferative effects of Api. Api treatment increased cell accumulation in the G2/M phases, but in the absence of cyclin B1, the cells did not have the necessary energy to transition from the G2 phase to the M phase.

Consequently, in response to Api treatment, the cells were arrested at the G2 phase. Our results regarding the effect of Api on the expression of FOXM1 are similar to the recently reported results obtained with compound NB-73, which is an inhibitor of FOXM1 [43]. In that study, Ziegler et al. showed that NB-73 decreased the expression of FOXM1 and simultaneously increased the accumulation of cells in the G2/M phases. Furthermore, the induction of G2/M phase cell cycle arrest by Api in MCF-7 cells was also recently documented by Shendge et al. [44]. According to those authors, this effect of Api is due to its induction of intracellular ROS production. Our findings regarding the action of Api through FOXM1 signaling are complementary to the findings regarding the effects of Api on the cell cycle.

The alteration in the cell cycle that caused the Api-induced G2/M phase cell cycle arrest may result in cell apoptosis. As expected, we showed that Api induced apoptosis in both the MCF-7/Ctrl cells and MCF-7/Akt cells. Our results were consistent with the results of other previous studies, in which Api was shown to induce apoptosis in different cancer cells, such as BC [24–28,35], ovarian cancer [45], and lung cancer [46]. Api exerts apoptotic effects through increases in the expression of p53 and the ratio of Bax/Bcl2, activation of caspases and cleavage of PARP [44–46]. Since the cell number is the result of cell proliferation, survival and cell death, the apoptotic effects of Api, accompanied by our results regarding the cell cycle, can explain the anti-proliferative effects of Api observed in our cell proliferation assays.

Together, in this study, we showed anti-proliferative effects of Api in Akt-activated MCF-7 breast cancer cells. The mechanisms of action of Api through ER signaling and Akt/FOXO3a/FOXM1 signaling were revealed. Notably, we observed a clear inhibitory effect of Api on FOXM1 and FOXM1 activity. Since FOXM1 is upregulated and over-expressed in aggressive therapy-resistant forms of breast cancer and is associated with worse prognosis of patients [43,47], Api warrants further study as a potential treatment for endocrine-resistant BC.

4. Materials and Methods

4.1. Reagents

Apigenin (Api) (A3145) $\geq 97\%$ (TLC) from parsley, 4-hydroxytamoxifen (4-OHT) (H7904) $\geq 98\%$ Z isomer, and β -estradiol (E2) (E8875) $\geq 98\%$ were purchased from Sigma-Aldrich (St Louis, MO, USA).

4.2. Cell Clone Establishment and Maintenance

The MCF-7 subclones were established using the T-Rex™ system (Thermo Fisher Scientific, Waltham, MA, USA), including the pcDNA6/TR and pcDNA4/TO plasmids. Ctrl and Akt pcDNA4/TO expression vectors were generated from the empty vector and myr-Akt1 cDNA (activated) vector pUSEamp (Upstate Cell Signaling Solutions, Lake Placid, NY, USA), respectively. Note that we had to mutate the PmeI site of the pcDNA4/TO plasmid to insert the myr-Akt1 cDNA vector. In detail, one of the two PmeI sites of the pcDNA4/TO plasmid was changed into a HpaI site by a direct mutation, and Myr-Akt1 cDNA was inserted between the HpaI and HindIII sites. Stably transfected MCF-7/Ctrl and MCF-7/Akt clones were obtained by transfecting MCF-7 cells with a pcDNA6/TR plasmid and corresponding pcDNA4/TO expression vectors with the jetPEI reagent (Polyplus Transfection, Illkirch, France). Zeocin (75 $\mu\text{g}/\text{mL}$; Thermo Fisher Scientific, Waltham, MA, USA) and blasticidin (5 $\mu\text{g}/\text{mL}$; Thermo Fisher Scientific, Waltham, MA, USA) were used as the selection antibiotics. The stable clones were maintained in Dulbecco's modified Eagle's medium (DMEM, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 4.5 g/L glucose, nonessential amino acids (NEAA, Thermo Fisher Scientific, Waltham, MA, USA), penicillin/streptomycin (Thermo Fisher Scientific, Waltham, MA, USA), 8% fetal bovine serum (FBS, Biowest, Nuaille, France) and the two selective antibiotics. All the cells were cultured at 37 °C in 5% CO₂. Before treatment, the cells were cultured in serum-free DMEM without phenol red or steroids and with 2% charcoal/dextran-stripping FBS (Biowest, Nuaille, France) and treated with 1 $\mu\text{g}/\text{mL}$ tetracycline to activate Akt.

4.3. Proliferation Assay

Cell proliferation was tested using the sulforhodamine B assay. A total of 3000 cells/well were seeded into 96-well plates. After 24 h, the cells were incubated with serum- and steroid-deficient medium and treated with 1 $\mu\text{g}/\text{mL}$ tetracycline for 48 h. The cells were treated with solvent (0.1% DMSO (*v/v*) and 0.1% ethanol (*v/v*)) as a control, 4-OHT, 10 μM Api alone or in combination with 4-OHT, in the presence or absence of 1 nM E2 for 6 days. At the end of the experiment, the cells were fixed using trichloroacetic acid (TCA) (T0699, Sigma-Aldrich, St Louis, MO, USA) for 1 h at 4 °C and then washed 4 times with tap water.

After air drying, the cells were stained with 0.04% (*w/v*) sulforhodamine B for 30 min and then washed 4 times with 1% (*v/v*) acetic acid. After air-drying one more time, the protein-bound dye was solubilized using the Tris base solution at 10 mM. The absorbance was measured at 490 nm. The cell numbers were determined using calibration ranges (Supplementary Figure S1).

4.4. Cell Cycle Analysis by Flow Cytometry (FACS)

A total of 1,000,000 cells per dish were plated in 10-cm dishes and incubated for 24 h. Then, the cells were deprived of steroids and serum for 24 h. Next, the cells were treated with solvent (0.1% DMSO (*v/v*) and 0.1% ethanol (*v/v*)) as a control, 4-OHT, 10 μ M Api alone or in combination with 4-OHT, in the presence or absence of 1 nM E2 for approximately 24 h. After treatment, the cells were trypsinized and fixed with 70% ethanol before being stained with propidium iodide (Sigma-Aldrich, St Louis, MO, USA) in the presence of RNase A. The cell cycle was analyzed with a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

4.5. Apoptosis Analysis

A total of 20,000 cells/well were grown on 10-mm-diameter coverslips in 24-well plates. After 24 h, the cells were incubated with serum- and steroid-deficient medium and treated with 1 μ g/mL tetracycline for 48 h. The cells were treated with solvent (0.1% DMSO (*v/v*) and 0.1% ethanol (*v/v*)) as a control, 4-OHT, 10 μ M Api alone or in combination with 4-OHT, in the presence or absence of 1 nM E2 for approximately 24 h. Terminal deoxynucleotidyl dUTP nick end labeling (TUNEL) staining was performed with an In Situ Cell Death Detection Kit, Fluorescein (Sigma-Aldrich, St Louis, MO, USA) according to the manufacturer's instructions. The percentage of TUNEL-positive cells was determined by using ImageJ software (NIH, Bethesda, MD, USA).

4.6. Protein Extraction and Western Blotting

A total of 300,000 cells/well were seeded into 6-well plates. After 24 h, the cells were incubated with serum- and steroid-deficient medium and treated with 1 μ g/mL tetracycline for 48 h. Then, the cells were treated with solvent (0.1% DMSO (*v/v*) and 0.1% ethanol (*v/v*)) as a control or 10 μ M Api alone or in combination with 1 nM E2 for 24 h. After the treatment, the cells were directly lysed in 2X Laemmli buffer. The protein extracts were denatured for 10 min at 95 °C, separated on 7.5% SDS polyacrylamide gels, and transferred to polyvinylidene difluoride membranes (Millipore). The proteins were then probed with specific antibodies. The following antibodies were used: anti-Akt1 (sc-5298, Santa Cruz Biotechnology, Dallas, TX, USA) (dilution 1:1000), anti-FOXM1 (D12D5, Cell Signaling Technology, Danvers, MA, USA) (dilution 1:1000), anti-FOXO3a (ab53287, Cambridge, MA, USA) (dilution 1:1000), anti-cyclin B1 (sc-245, Santa Cruz Biotechnology, Dallas, TX, USA) (dilution 1:1000), anti-FOXO1 (C29H4, Cell Signaling Technology, Danvers, MA, USA) (dilution 1:1000) and anti- β -actin (A1978, Sigma-Aldrich, St Louis, MO, USA) (dilution 1:5000), which served as a control for the total amount of protein. An enhanced chemiluminescence system (Immune-Star, Bio-Rad, Hercules, CA, USA) was used to detect the immunocomplexes.

4.7. Luciferase Assay

A total of 40,000 cells/well were seeded into 24-well plates. After 24 h, the cells were incubated with serum- and steroid-deficient medium and treated with 1 μ g/mL tetracycline for 48 h. The cells were transfected using JetPEI (Polyplus Transfection) overnight with an artificial promoter containing an ERE upstream of the TK promoter (ERE-Luc), AP1-Luciferase (AP1-Luc), or Sp1-Luciferase (Sp1-Luc). As a control for the transfection efficiency, a CMV- β galactosidase vector was used. The following day, the cells were treated with solvent (0.1% DMSO (*v/v*) and 0.1% ethanol (*v/v*)) as a control or 10 μ M Api, alone or in combination with 1 nM E2 for 24 h. Then, reporter lysis buffer

(Promega, Madison, WI, USA) was used to lyse the cells, and the luciferase activity was measured with a commercial luciferase assay system (Promega, Madison, WI, USA). The luciferase activity was normalized to β -galactosidase activity. The data are presented as the fold change relative to the control condition.

4.8. Immunofluorescence

A total of 20,000 cells/well were grown on 10-mm-diameter coverslips in 24-well plates. After 24 h, the cells were incubated with serum- and steroid-deficient medium and treated with 1 μ g/mL tetracycline for 48 h. Then, the cells were treated with solvent (0.1% DMSO (*v/v*) and 0.1% ethanol (*v/v*)) as a control and 10 μ M Api alone or in combination with 1 nM E2 for 24 h. After treatment, the cells were fixed with 4% formaldehyde solution/neutral buffered saline (Sigma-Aldrich, St Louis, MO, USA) for 15 min and then permeabilized in PBS/3% Triton X-100 for 10 min. Next, the cells were unmasked with 50 mM Tris-HCl pH 9.5 at 80 °C for 30 min. The cells were incubated overnight at 4 °C with primary antibodies against phospho-Bad (Ser112, Cell Signaling Technology, Danvers, MA, USA) (dilution 1:1000), phospho-mTOR (Ser2448, Cell Signaling Technology, Danvers, MA, USA) (dilution 1:1000), FOXO3a (ab124394, Abcam, Cambridge, MA, USA) (dilution 1:1000) or FOXM1 (D12D5, Cell Signaling Technology, Danvers, MA, USA) (dilution 1:1000) and then with a secondary antibody (dilution 1:1000) at room temperature for one hour. The coverslips were mounted with Duolink[®] in situ mounting medium with 4',6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, St Louis, MO, USA) before microscopic analysis.

4.9. Nuclear/Cytoplasmic Ratio Quantification from Immunofluorescence Images

ImageJ software (NIH, Bethesda, MD, USA) was used to create a custom macro to generate an automated tool to quantify the nuclear/cytoplasmic fluorescence ratio of each cell in the images. The first mask, corresponding to the nuclear area, was determined using DAPI fluorescence. The second mask, which was considered to be the cytoplasmic area, is created by homothetic enlargement of the first mask to generate a ring around the nucleus. These two masks were successively applied to quantify the fluorescence of the protein of interest in the nucleus and in the cytoplasmic area. Then, the nuclear/cytoplasmic immunofluorescence ratio was calculated for each cell. The median of the nuclear/cytoplasmic ratio of the control condition was used as a threshold to determine the percentage of cells below and above this threshold in each condition. For each condition, approximately 1000–1500 cells from at least 10 images were analyzed.

4.10. RNA Extraction and Real-Time PCR

A total of 300,000 cells/well were plated in 6-well plates. After 48 h of serum and steroid deprivation and tetracycline treatment, the cells were treated with solvent (0.1% DMSO (*v/v*) and 0.1% ethanol (*v/v*)) as a control and 10 μ M Api alone, or in combination with, 1 nM E2 for 24 h. The total RNA was obtained using a mini RNeasy kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Then, using an M-MLV RT kit (Thermo Fisher Scientific, Waltham, MA, USA), the RNA was reverse-transcribed according to the manufacturer's instructions. For real-time PCR, 5 ng of cDNA was used with 150 nM primers (Table 1) and iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, USA). Real-time PCR was carried out in a CFX 384 apparatus, and the data were analyzed with CFX Manager Software (Bio-Rad, Hercules, CA, USA).

Table 1. Gene names and primer sequences used in this study.

Gene Name and Symbol	Forward Primer	Reverse Primer
GATA-binding protein 3 (GATA3)	GCCGTTGAGGGTTTCAGAGA	TCCGAGCACAACCACCTTAG
Forkhead box A1 (FOXA1)	CCCCTTTGTCCTCTCTACCC	CTGCAAAGCAAGAAGCAGAGT
Estrogen receptor 1 (ESR1/ER α)	TTTATGGGAAAAGGCTCAAA	GACAAAACCGAGTCACATCA
Chemokine (C-X-C motif) ligand 12 (CXCL12)	CACCATTGAGAGGTCGGAAG	AATGAGACCCGTCCTTTCAG
Progesterone receptor (PgR)	CCCGCCGTCGTAACCTTGG	GTGCCTATCCTGCCTCTCAATC
Amphiregulin (AREG)	GTATTTTCACTTTCGGTCTTGTTTTG	CCTGGCTATATTGTTCGATTCA
Forkhead box M1 (FOXM1)	AGTGCCAACCGCTACTTGAC	CACCGGGAAGTGGATAGGTA
Cell division cycle 25A (CDC25A)	CAAGGGTGCAGTGAACCTTGC	ACAACAATGACACGCTTGCC
Cyclin B1 (CCNB1)	TCTGGATAATGGTGAATGGACA	CGATGTGGCATACTTGTCTTG
Centromere protein A (CENPA)	ACATGCAGCCGAGTTACTC	AGAGTCCCCGGTATCATCCC
Polo-like kinase 1 (PLK1)	CTCAACACGCCTCATCCTC	GTGCTCGCTCATGTAATTGC
Cyclin-Dependent kinase inhibitor 1A (CDKN1A/p21)	CTGTCTTGTACCCTTGTGCC	GGTAGAAATCTGTCATGCTGG
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG
TATA box-binding protein (TBP)	TGCACAGGAGCCAAGAGTGAA	CACATCACAGCTCCCCACCA

4.11. Statistical Analyses

Statistical significance was quantified with one-way analysis of variance (ANOVA) and Tukey's post hoc test or with a Mann-Whitney test using GraphPad Prism software (version 5, GraphPad Software, San Diego, CA, USA).

Supplementary Materials: The following are available online at <https://www.mdpi.com/1422-0067/22/1/470/s1>, Figure S1: Calibration ranges of cell number and absorbance tests by sulforhodamine B assay, Figure S2: Nuclear/cytoplasmic fluorescence intensity ratio of FOXM1, Figure S3: Equal amounts of protein extracts from MCF-7/Ctrl cells and MCF-7/Akt cells were analyzed by Western blotting with antibodies specific for FOXO1 and β -actin.

Author Contributions: T.H.P. and F.P. (Farzad Pakdel) conceived the experiments and were the major contributors to writing the original draft of the manuscript. T.H.P., Y.L.P., G.F. and F.P. (Farzad Pakdel) performed the experiments and analyzed the data. F.P. (Frédéric Percevault) constructed the MCF7/Cont and MCF7/Akt clones. F.P. (Farzad Pakdel), T.H.P., G.F. and F.F. reviewed and edited the manuscript. F.P. (Farzad Pakdel) supervised and coordinated the study. All authors have read and agreed to the published version of the manuscript.

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Abbreviations

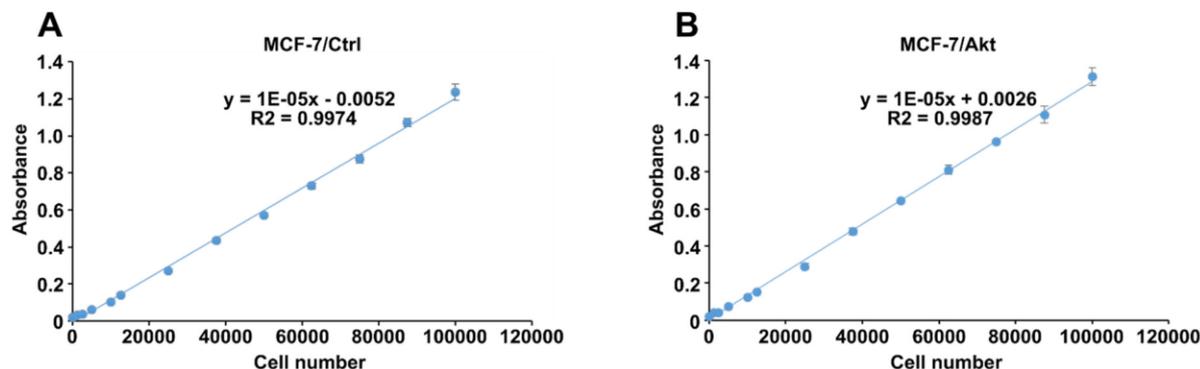
4-OHT	4-Hydroxytamoxifen
Api	Apigenin
BC	Breast cancer
E2	Estradiol
FOXM1	Forkhead box protein M1
TNBC	Triple-negative breast cancer

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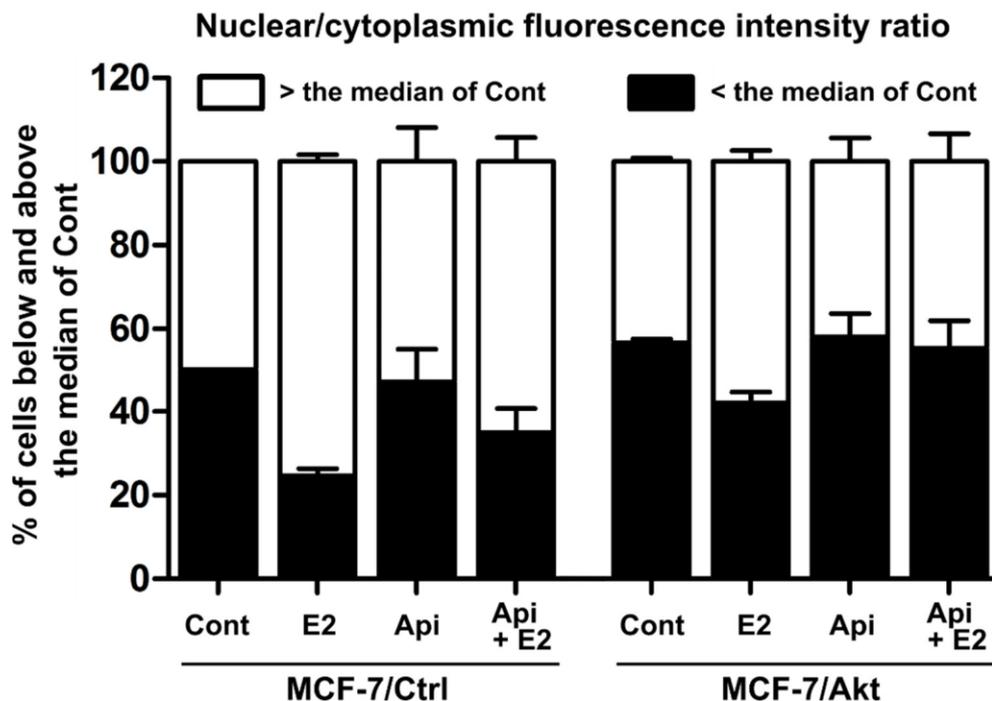
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Supplementary Figure S1

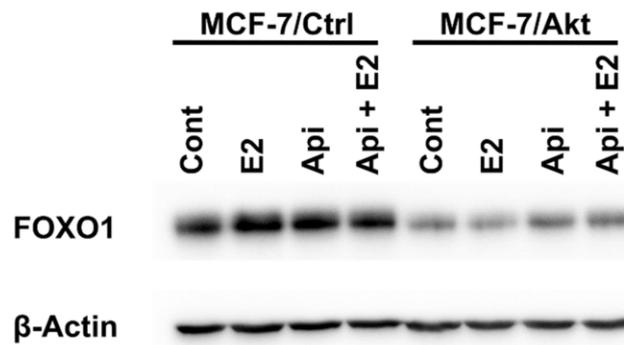


Supplementary Figure S1. Calibration ranges of cell number and absorbance tests by sulforhodamine B assay. MCF-7/Ctrl cells (A) and MCF-7/Akt cells (B) were seeded into 96-well plates and incubated for 6 h. Then, the cells were fixed and stained with sulforhodamine B. The absorbance of the protein-bound dye was measured at 490 nm. The results showed that the relation between cell number and absorbance followed a linear function. The linear equation and coefficient of determination are indicated in each graph.

Supplementary Figure S2



Supplementary Figure S2. Nuclear/cytoplasmic fluorescence intensity ratio of FOXM1. MCF-7/Ctrl cells and MCF-7/Akt cells were treated with 0.1% (v/v) DMSO and 0.1% (v/v) ethanol as the control (Cont), 1 nM estradiol (E2), or 10 μ M apigenin (Api) alone or in combination with E2 for 24 h. The fluorescence intensities in the nucleus and cytoplasm of each cell was measured using ImageJ software. The median of the nuclear/cytoplasmic ratio of the control condition was used as a threshold to determine the percentage of cells below and above this threshold in each condition. For each condition, approximately 1000-1500 cells from at least 10 images were analyzed. The experiment was conducted 3 times. The data are presented as the mean \pm SEM.

Supplementary Figure S3

Supplementary Figure S3. Equal amounts of protein extracts from MCF-7/Ctrl cells and MCF-7/Akt cells were analyzed by Western blotting with antibodies specific for FOXO1 and β -actin. MCF-7/Ctrl cells and MCF-7/Akt cells were treated with solvent (Cont), E2 and Api alone or in combination with E2 for 24 h

DISCUSSION ET PERSPECTIVES

Tendances émergentes de l'étude des molécules naturelles

Actuellement, une reprise d'intérêt existe pour des produits naturels et leurs impacts sur la santé, la médico-nutrition et l'économie [339]. Des progrès en phytochimie (isolation, purification), en informatique et en méthodologie de recherche biologique et pharmacologique nous permettent aujourd'hui d'étudier des molécules naturelles d'une manière conceptuelle et rationnelle. Cela a conduit à la caractérisation d'effets biologiques des molécules issues de plantes qui sont, dans beaucoup de cas, utilisées depuis longtemps dans la médecine traditionnelle. De plus, la recherche de médicaments efficaces issus de sources naturelles et présentant moins d'effets secondaires indésirables que des médicaments synthétiques, stimule l'étude de telles molécules. Actuellement, environ 50% des médicaments anticancéreux approuvés au niveau international sont soit des produits naturels, soit leurs dérivés [340]. Des effets anti-cancéreux ont été rapportés pour des molécules provenant de diverses sources naturelles telles que les taxanes (paclitaxel, docétaxel), les alcaloïdes de la vinca (vincristine, vinblastine, vindésine, vinorelbine), les podophyllotoxines et ses dérivés (étoposide, téniposide); les camptothécines et ses dérivés (topotécan, irinotécan); les anthracyclines (doxorubicine, daunorubicine, épirubicine, idarubicine) [340]. Les plantes sont un réservoir important de molécules potentiellement thérapeutiques et représentent une piste importante pour la recherche médicale.

En suivant ces tendances, ce travail de thèse a mis en évidence des effets anti-cancéreux des glycéollins et de l'apigénine dans le CS avancé. Les glycéollins, métabolites secondaires trouvés dans le soja, ont des effets anti-migration dans les cellules cancéreuses mammaires triple-négatives. L'apigénine, flavone existant dans un grand nombre de plantes, inhibe la prolifération des cellules cancéreuses mammaires Akt-activées et hormono-résistantes.

Nouvelles cibles thérapeutiques du CS

Bien que le CS soit largement étudié et que de gros progrès dans le diagnostic et les traitements aient vu le jour, il reste le cancer le plus fréquent chez les femmes dans le monde entier [12]. La majorité des tumeurs du sein humain expriment ER α et dépendent principalement des œstrogènes pour leur croissance [9]. Actuellement, l'agent de première ligne pour le traitement du cancer du sein ER+, aussi bien chez les femmes pré-ménopausées que chez les femmes ménopausées, est l'anti-œstrogène tamoxifène. Les thérapies endocriniennes tels que le tamoxifène et le fulvestrant ont considérablement réduit les récurrences et la mortalité causées par le CS, la résistance *de novo* et acquise à ce traitement reste, cependant, un défi majeur [60]. Pour des tumeurs résistantes et/ou à des stades avancés ou agressifs, les choix de traitement sont limités et la maladie peut être mortelle [9]. C'est pourquoi il est indispensable de découvrir de nouvelles cibles thérapeutiques et de trouver des agents qui peuvent réagir sur ces cibles. Dans notre étude, nous ciblons deux voies de signalisation émergentes impliquées dans le CS avancé.

Initialement connu comme un médiateur de la toxicité de TCDD, AhR est considéré actuellement comme une cible intéressante dans le CS [126,129,154]. Plusieurs ligands d'AhR, à la fois naturels et synthétiques, ont montré des effets anticancéreux à travers la signalisation d'AhR [146,155–168]. Dans notre étude, nous avons montré que GI et GII sont des ligands d'AhR et sont capables d'inhiber la migration cellulaire. Cependant, le rôle d'AhR dans le cancer dépend du type de cancer, de son stade d'avancement et du ligand d'AhR étudié [341]. En conséquence, des études plus approfondies sont nécessaires pour mieux comprendre le rôle de ce facteur de transcription dans le cancer. Ces connaissances pourraient faciliter l'étude des effets de ses ligands dans le cancer.

Une autre cible que nous avons étudiée dans cette thèse est Akt, la protéine centrale dans la voie de signalisation PI3K/Akt/mTOR. L'hyperactivation des protéines de cette voie, dont Akt, favorise la survie des cellules cancéreuses du sein

et entraîne une résistance aux thérapies endocriniennes [114,115]. Nous avons établi des cellules cancéreuses mammaires Akt-activées. La prolifération basale de ces cellules est plus importante que les cellules contrôles, moins dépendante de l'E2 et partiellement résistante au traitement par le 4-OHT (métabolite actif du tamoxifène, utilisé en clinique). Ce modèle nous a permis d'étudier l'effet de l'apigénine sur la prolifération des cellules Akt-activées en traitant seule ou en combinaison avec le 4-OHT. Les mécanismes d'action de l'apigénine dans ce contexte ont été également étudiés.

Glycéollins : Discussion et perspectives

Bien que les effets anti-œstrogéniques des glycéollins aient été mis en évidence dans les années 2000 [342], il reste beaucoup à faire pour mieux comprendre leurs effets thérapeutiques sur le cancer, le diabète, les maladies cardio-vasculaires et neurodégénératives. Dans notre étude, nous avons mis en évidence, pour la première fois, l'activité des glycéollins sur la voie de signalisation d'AhR et leur effet inhibiteur sur la migration des cellules cancéreuses mammaires triple-négatives.

Les modèles cellulaires sont privilégiés pour faire un criblage des molécules ou pour étudier des mécanismes d'action. Cependant, l'absence des réelles conditions physiologiques d'un être vivant est une faiblesse importante de ces modèles. Il est donc indispensable de confirmer les effets des glycéollins sur les modèles *in vivo*. Salvo *et al.* ont montré que les glycéollins présentent des effets anti-prolifératifs sur des tumeurs mammaires xéno greffées chez la souris nude lorsqu'ils sont administrés par injection [343]. Cependant, l'administration par injection est souvent plus compliquée et coûteuse que l'administration orale. En utilisant le même modèle, notre équipe a entrepris une étude sur les effets des glycéollins apportés par voie orale (publication en cours de préparation). De plus, à la différence des études précédentes où les glycéollins étaient utilisés sous forme individuelle GI ou GII synthétisée pure, les souris sont traitées avec de l'extrait naturel de soja enrichi en glycéollins préparé sous forme de bonbons.

Si les effets anti-prolifératifs des glycéollins sont confirmés chez ces souris, nous voulons, dans un deuxième temps, tester les effets anti-migration des glycéollins *in vivo*. Un exemple de modèle envisageable est d'injecter des cellules MDA-MB-231 dans la veine de la queue des souris pour ensuite étudier les métastases au niveau du poumon.

En plus de notre équipe, plusieurs autres équipes scientifiques internationales continuent à caractériser les propriétés biologiques des glycéollins et s'y intéressent sous différents aspects. Par exemple, une équipe japonaise vient de démontrer chez le rat que les glycéollins sont plus absorbables que l'isoflavone daidzéine [344]. Cela est dû à leur forte hydrophobicité et au métabolisme vers les formes plus absorbables des glycéollins au cours du processus d'absorption intestinale. Ces résultats sont encourageants pour les équipes qui veulent développer les glycéollins comme un traitement par voie orale. Afin d'avoir une quantité de glycéollins suffisante pour que le public puisse bénéficier de ces produits, la recherche sur la production s'impose. Il y a récemment des progrès concernant l'isolation et la production des glycéollins. Ciesielski et Metz viennent de découvrir une voie de synthèses asymétriques des composés naturels [345]. Ils ont ensuite adapté avec succès cette voie à la première synthèse totale asymétrique catalytique de (-) - GI et (-) - GII. Nos collaborateurs dans le projet mVolio – FUI ont réussi à développer une approche innovante qui permet de transformer la majeure partie des isoflavones de soja en glycéollins, en utilisant la fermentation en milieu solide.

Apigénine : Discussion et perspectives

Contrairement aux glycéollins qui sont seulement produits lors de l'exposition de soja aux éliciteurs, l'apigénine est présente dans notre alimentation [197]. Du fait que la population est largement exposée à cette molécule, il est nécessaire de comprendre ses effets biologiques. De plus, l'apigénine est déjà commercialisée seule ou avec d'autres flavonoïdes pour utiliser comme compléments alimentaires préventifs de certaines maladies comme le CS ou pour améliorer le bien-être ou dans

la cosmétique. Nous pouvons trouver sur le marché les compléments alimentaires de Swanson, SD Pharmaceuticals, Kenayeuro et Cosomed qui contiennent l'Api sous forme de capsule de 50 ou 40 mg.

Nos résultats antérieurs ont montré que l'apigénine a une action anti-proliférative sur deux modèles de carcinome mammaire humain, les lignées MCF-7 et T47D traitées à l'E2. Bien que l'apigénine régule un ensemble similaire de gènes régulés également par l'E2, la dynamique transcriptionnelle des gènes induite par l'apigénine est différente de celle induite par l'E2 [195]. Dans notre étude plus récente, nous avons montré que l'apigénine inhibe également la prolifération des cellules cancéreuses mammaires ER-positives Akt-activées. Il serait alors pertinent d'analyser le transcriptome dans ces cellules. Il serait également intéressant de tester l'effet de l'apigénine sur les cellules PI3K-activées qui sont en train d'être développées dans l'équipe. Le co-traitement avec l'apigénine a augmenté l'efficacité du 4-OHT. Il serait donc intéressant d'explorer l'action combinée avec d'autres molécules utilisées dans le traitement du CS en hormonothérapies (des SERMs ou des inhibiteurs de l'aromatase) ou en chimiothérapies (les agents alkylants ou les taxanes) et de voir s'il est possible de diminuer la dose de ces molécules et ainsi de limiter les effets secondaires des traitements.

A travers les études internationales, l'apigénine a montré son potentiel anti-cancéreux non seulement dans le CS mais aussi d'autres cancers : poumon, foie, peau, sang, côlon, prostate, pancréas, cou, bouche et estomac en modulant plusieurs voies de signalisation [209]. Cependant, la plupart des études ont été faites *in vitro* [209]. Le dosage et l'efficacité de l'apigénine contre les lignées cellulaires cancéreuses ne peuvent pas être comparés aux systèmes vivants, car ces derniers sont plus complexes. Certaines études, basées sur des modèles animaux, valident également le pouvoir inhibiteur de l'apigénine sur le développement tumoral, mais ces études ont souvent des limites à cause du petit nombre d'animaux testés [209]. Pour cette raison, les études approfondies, notamment les études cliniques, sont demandées avant que cette molécule puisse devenir un traitement contre le cancer.

Pistes pour étudier les molécules naturelles

Bien qu'on comprenne de mieux en mieux les modes d'action des molécules naturelles, dont les glycéollins et l'apigénine, notre connaissance sur ces composés reste encore très limitée. Cela ouvre plusieurs pistes pour la recherche.

Découverte et production : Le règne végétal contient environ 310 000 espèces et chaque espèce peut produire une large gamme de composés bioactifs [346]. La plupart de ces composés reste à découvrir. L'identification des nouveaux composés, des nouvelles cibles thérapeutiques des molécules déjà existantes ou des nouvelles combinaisons est une piste importante. Ce travail est de plus en plus facilité par les progrès de la chimie computationnelle. En ce qui concerne la production, davantage de recherches sont en cours pour optimiser la production, améliorer la qualité et réduire le coût des molécules naturelles afin de les rendre accessibles [346].

Forme d'administration : Malgré leurs effets biologiques prouvés, de nombreuses molécules naturelles ont une faible biodisponibilité orale [347]. Pour pouvoir bénéficier de ces molécules, il est primordial de trouver une forme galénique adaptée qui peut augmenter sa biodisponibilité. Par ailleurs, la médecine moderne utilise des molécules naturelles plutôt sous forme seule, alors que la médecine traditionnelle, notamment la médecine traditionnelle chinoise, les utilise en formules [348]. Ces dernières sont constituées d'un mélange de molécules dont certaines ont un rôle d'excipients qui peuvent augmenter l'efficacité des molécules principales et diminuer les effets secondaires [348]. Il est donc intéressant d'étudier les molécules naturelles en combinaison. Une des stratégies de cette direction est de les utiliser sous forme d'extrait. Cette forme permet d'ailleurs de simplifier la procédure de production. Dans ce cas, par exemple pour l'extrait naturel de soja enrichi en glycéollins, il est important de définir et d'authentifier la composition chimique de l'extrait proposé. De plus, la toxicité de non seulement les molécules actives mais aussi de l'extrait entier doit être testée avant l'utilisation chez les humains.

Etude Clinique: Plusieurs molécules naturelles sont commercialisées sous formes de compléments alimentaires en se basant seulement sur les résultats obtenus *in vitro* et *in vivo* [209]. Cela pose des questions sur leur efficacité et leur innocuité chez l'humain. Comme abordé dans la discussion sur l'apigénine, les études cliniques seraient primordiales pour que les molécules naturelles puissent être utilisées comme médicaments.

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ANNEXES

Synthèse de la publication N°4: “Deciphering the Molecular Mechanisms Sustaining the Estrogenic Activity of the Two Major Dietary Compounds Zearalenone and Apigenin in ER-Positives Breast Cancer Cell Lines”

La flavone apigénine et la mycotoxine zéaralénone sont largement retrouvées dans notre alimentation. Dans une étude de criblage de plusieurs molécules naturelles, notre équipe a trouvé que ces deux molécules pouvaient transactiver ER dans différentes lignées cellulaires [193]. Cependant, leurs effets phénotypiques sur les cellules sont opposés. Tandis que l'apigénine est un faible inducteur de la prolifération cellulaire cancéreuse mammaire et un bon potentialisateur de la différenciation neuronale, la zéaralénone est un puissant inducteur de la prolifération cellulaire cancéreuse mammaire et toxique pour la différenciation neuronale. Notre étude a pour objet d'élucider les mécanismes qui peuvent expliquer les effets différents entre l'apigénine et la zéaralénone dans le contexte de cellules cancéreuses ER-positives. Pour atteindre ce but, les essais de gène-rapporteur, d'immunoprécipitation de la chromatine (ChIP), de prolifération cellulaire, de PCR quantitative et d'analyse transcriptomique ont été effectués.

Nous avons constaté que les deux molécules ont transactivé ER α bien qu'une différence dans la cinétique de l'activité de la luciférase ait été observée. Les expériences ChIP n'ont montré aucune différence majeure dans le recrutement d'ER α vers divers sites de liaison d'ER lorsque l'ER α était activé par la zéaralénone ou l'apigénine. Les deux molécules ont favorisé l'expression de gènes cibles de l'estradiol (E2), mais la zéaralénone est plus puissante. Concernant la prolifération cellulaire, dans les deux lignées cellulaires de cancer du sein ER-positives, MCF-7 et T47D, la zéaralénone a provoqué un effet prolifératif complet et puissant en l'absence ou en présence d'E2, tandis que l'apigénine a montré un effet prolifératif partiel et faible en l'absence d'E2 et un effet antagoniste en présence d'E2. L'analyse transcriptomique a montré que les deux composés régulent l'expression des gènes

de la même manière, mais avec des différences d'intensité. Deux ensembles majeurs de gènes modifiés par la zéaralénone et l'apigénine ont été identifiés; l'un était lié au cycle cellulaire et l'autre à la réponse au stress et à l'arrêt de la croissance. Nos résultats montrent que la dynamique de transcription dans la régulation des gènes induite par l'apigénine était quelque peu différente avec la zéaralénone et E2 et peut expliquer l'effet différentiel de ces composés sur le phénotype de la cellule de CS. En conclusion, nos résultats ont confirmé l'effet bénéfique potentiel de l'apigénine sur le CS, alors que la zéaralénone semble être un véritable perturbateur endocrinien.

PUBLICATION N°4 « Deciphering the Molecular Mechanisms Sustaining the Estrogenic Activity of the Two Major Dietary Compounds Zearalenone and Apigenin in ER-Positive Breast Cancer Cell Lines »

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Article

Deciphering the Molecular Mechanisms Sustaining the Estrogenic Activity of the Two Major Dietary Compounds Zearalenone and Apigenin in ER-Positive Breast Cancer Cell Lines

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Abstract: The flavone apigenin and the mycotoxin zearalenone are two major compounds found in the human diet which bind estrogen receptors (ERs), and therefore influence ER activity. However, the underlying mechanisms are not well known. To unravel the molecular mechanisms that could explain the differential effect of zearalenone and apigenin on ER-positive breast cancer cell proliferation, gene-reporter assays, chromatin immunoprecipitation (ChIP) experiments, proliferation assays and transcriptomic analysis were performed. We found that zearalenone and apigenin transactivated ERs and promoted the expression of estradiol (E2)-responsive genes. However, zearalenone clearly enhanced cellular proliferation, while apigenin appeared to be antiestrogenic in the presence of E2 in both ER-positive breast cancer cell lines, MCF-7 and T47D. The transcriptomic analysis showed that both compounds regulate gene expression in the same way, but with differences in intensity. Two major sets of genes were identified; one set was linked to the cell cycle and the other set was linked to stress response and growth arrest. Our results show that the transcription dynamics in gene regulation induced by apigenin were somehow different with zearalenone and E2 and may explain the differential effect of these compounds on the phenotype of the breast cancer cell. Together, our results confirmed the potential health benefit effect of apigenin, while zearalenone appeared to be a true endocrine-disrupting compound.

Keywords: dietary compounds; estrogen receptor; breast cancer; gene expression; endocrine disruption

1. Introduction

The human population is commonly exposed to numerous estrogenic compounds through the environment, including the diet. Epidemiological studies estimate that exposure to these environmental chemicals may potentially impact human and animal health [1]. These approaches provided statistical associations between environmental estrogens and fertility or various cancers, particularly breast cancer [2]. Some of these statistical associations, notably, the association with soy consumption, showed beneficial effects [3,4]. Among these environmental estrogens, some are chemicals such as pesticides or insecticides; others are naturally produced by plants or fungi. This is the case with the well-documented flavones, isoflavones, stilbenes, mycotoxins, and so on. In a previous

study, we screened the impact of different compounds produced by plants and belonging to different chemical classes on two cellular phenotypes, namely, cellular proliferation and cellular differentiation. From this screen, we identified zearalenone and apigenin, which had opposite biological effects. Indeed, we had shown that zearalenone and apigenin were able to transactivate ERs in different cell models and regulated ER-gene expression such as chemokine (C-X-C motif) ligand 12 (CXCL12). Moreover, zearalenone was found as a strong inducer of cellular proliferation and toxic for neuronal differentiation while apigenin was a weak cellular proliferation inducer and a good potentiator of neuronal differentiation [5].

Zearalenone is a mycotoxin mainly produced by *Fusarium* fungi as a second metabolite and is reported to have adverse effects on health. Particularly, zearalenone has been associated with cancer, immunotoxicity, reprotoxicity, and advanced puberty, probably via its interaction with estrogen receptors (ERs) [6]. Because zearalenone is found in corn, wheat, barley, sorghum and rye, the human population is exposed directly through the consumption of contaminated cereals or indirectly through livestock fed contaminated cereals. Moreover, zearalenone is resistant to food processing methods such as heating. Currently, the advisory level for zearalenone is not defined in the USA, while it is limited to 20–100 µg/kg for cereals and up to 400 µg/kg for maize in Europe, with a tolerable daily intake of 0.25 µg/kg body weight. A recent work assessed the level of circulating zearalenone and its metabolites in a group of 48 women. Free zearalenone was detected in serum from 85.4% of the participants, with a mean concentration of 0.026 ± 0.022 ng/mL [7].

Unlike zearalenone, the flavone apigenin, which is found in numerous plant foods, is described as having beneficial effects on human health, particularly on cancer [8]. Indeed, apigenin is present in teas and dry herbs, particularly chamomile flowers (395–1200 mg/100 g dry weight) and parsley (up to 1500 mg/100 g fresh weight), and in several other foods such as juices, fruits, honey and cereals [9]. It was estimated that women in the Nurses' Health Study ingested up to 1.3 mg/day of apigenin [10]. Pharmacokinetics studies were performed using aglycone form of apigenin. For instance, after oral administration of radiolabeled apigenin or the flower extract of *Daphne genkwa*, a plasma concentration peak was obtained after 1 h and reached approximately 40–50 ng/mL in rats. Moreover, these studies showed the sustained persistence of apigenin, suggesting an accumulation in tissues [11,12]. However, in humans, after consumption of parsley or chamomile extract, the plasma concentration of apigenin remains low (<1 µM), possibly due to its lower bioavailability in humans than in rodents and different metabolism by the intestinal microbiota [9,13]. Like zearalenone, apigenin binds to ERs and is able to transactivate them [5].

ERs belong to the nuclear receptor superfamily and are encoded by two distinct genes. ER α is the main regulator of estrogen-dependent genes. ER β , when it is coexpressed with ER α , has a tendency to restrain ER α activity [14]. In cells, ERs bind directly to DNA on estrogen-responsive elements (EREs) or interact with other transcription factors, such as specificity protein 1 (SP1) or activator protein 1 (AP1). They are also able to modulate intracellular pathways such as the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) pathways. ERs are extensively distributed in both male and female organisms, where they exert pleiotropic effects [15,16]. Estradiol (E2) is the natural hormone that binds ERs and is involved in the development and maintenance of the sexual organs in women. For instance, E2 is involved in breast epithelial cell proliferation and survival and, unfortunately, in breast cancer initiation and growth [17]. Breast cancer is the most prevalent cancer in women worldwide, with an incidence of approximately 90 cases per 100,000 women in developed countries. Breast cancer is ranked by the WHO as the fifth overall cause of death from cancer (522,000 deaths) [18]. ER-positive breast cancer represents approximately 80% of cases; therefore, ERs, particularly ER α , are considered good prognostic factors and are prime targets for therapy.

The aim of this study was to examine ER-mediated activation of cell proliferation by zearalenone and apigenin and to try to decipher mechanisms that sustained the differential activity of these compounds. In our previous work [5], we have used two ER-positive breast cancer cell lines (MCF-7

and T47D) to study the proliferative effect of these compounds. The results showed that the effect of zearalenone and apigenin was quite similar in these two cell lines. Zearalenone showed full estrogenic activity by stimulating cell proliferation, while apigenin exhibited partial agonistic activity, increasing cell numbers slightly. Thus, in this study, we confirmed our previous data on MCF-7 and T47D cell proliferation and showed the combined effect of zearalenone and apigenin with estradiol. However, the mostly of molecular analysis were performed on the MCF-7 cell line which is the main representing of luminal A breast cancer [19]. Luminal A and luminal B breast cancer belong to the ER-positive breast cancer family, but luminal A possesses more ER α than luminal B. Consequently, it is a reasonable model to study the estrogenic activity of compounds. We confirmed that both compounds activate ER α and ChIP assays on different ER binding sites clearly indicated that the actions of apigenin and zearalenone did not reside in the ability of these compounds to induce ER-DNA binding. However, these compounds have distinct effects on proliferation. By transcriptomic analysis, we found that the differential regulation of genes in response to zearalenone treatment perfectly mimicked the response to E2, while apigenin induced a distinct set of transcriptional response genes. More precisely, compared to gene transcription in response to E2 and zearalenone, the transcription of E2-target genes involved in cell growth was much less stimulated by apigenin, and, inversely, the transcription of those involved in cell growth arrest and apoptosis were much more stimulated (or less repressed) by apigenin.

2. Materials and Methods

2.1. Cell Culture and Reagents

MCF-7 cells were obtained from Jérôme Eeckhoutte (Dana Farber Cancer Institute) and T47D cells were purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (MCF-7) or Roswell Park Memorial Institute (RPMI) 1640 Medium (T47D) containing 4.5 g/L glucose supplemented with nonessential amino acids, penicillin/streptomycin (Invitrogen) and 10% fetal bovine serum (FBS) (Biowest) at 37 °C under 5% CO₂. For steroid treatment, cells were cultured for at least 24 h in steroid- and serum-deprived DMEM without phenol red and with 1.5% charcoal/dextran-stripped FBS (Biowest). Estradiol (E2), apigenin, and zearalenone were purchased from Sigma Aldrich (St Louis, MI, USA). ICI_{182,780} was purchased from Tocris Bioscience (Bristol, UK).

2.2. Luciferase Assay

First, 50,000 cells per wells were plated in 24-well multiwell plates. After serum and steroid deprivation, cells were transfected overnight with 100 ng of an estrogen responsive element-thymidine kinase (ERE-TK)-luciferase vector, which codes for luciferase under the control of one ERE, and with 50 ng of a cytomegalo virus (CMV)- β galactosidase vector, which served as a transfection efficiency control, using JetPEI (Polyplus Transfection, Illkrich, France). Next, cells were treated with solvent as a control, 10⁻⁹ M E2 or various doses of apigenin or zearalenone for different time periods. ICI_{182,780} at a concentration of 10⁻⁶ M was used as an ER inhibitor to test the specificity of ER transactivation. Cells were lysed in Passive Lysis Buffer (Promega, Madison, WI, USA), and luciferase activity was determined using a luciferase assay system (Promega, Madison, WI, USA).

2.3. Chromatin Immunoprecipitation

Cells were treated with solvent as a control, with 10⁻⁹ M E2, 10⁻⁸ M zearalenone or 10⁻⁵ M apigenin for 1 h and chromatin immunoprecipitation was performed as described in supplementary files of [20]. In this study the anti-ER α (HC20, Santacruz, Dallas, TX, USA) was used.

An enrichment analysis of the proximal ERE of GREB1 (Fwd: CACTTTGAGCAAAAGCCACA and Rev: GACCCAGTTGCCACACTTTT), on the enhancer of CUE domain containing 1 (CUEDC1) (Fwd: AGCATTGGTAAAGTCAGGCT and Rev: AGGAAGCTGGTGTCTTGGC), on enhancer 1 of x-box binding protein 1 (XBP1) (Fwd: TCACAGGCTGCCAAGTATCT and Rev:

TGGCGTAATTCAAACCCTGC) and on enhancer 2 of XBP1 (Fwd: AAACAATAGCCCAGAAGCCC and Rev: AGTCCAAGGGCACATTCTCA) was performed by real-time polymerase chain reaction PCR on a CFX384 Touch system (Bio-Rad) with 2 μ L of immunoprecipitation product or 0.2 μ L of input DNA, 500 nM primers, and iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, USA).

2.4. RNA Extraction and Real-Time PCR

Cells were treated with solvent as a control, with 10^{-9} M E2 or with various concentrations of apigenin or zearalenone as indicated in the legend figures. RNA extraction and real time PCR were performed as described in [20]. The sequence of the primers used in this study are described in Table 1.

Table 1. Gene names and primer sequences used in the real-time polymerase chain reaction (PCR) experiments.

Gene Name and Symbol	Forward Primer	Reverse Primer
Chemokine (C-X-C motif) ligand 12 (CXCL12)	CACCATTGAGAGGTCGGAAG	AATGAGACCCGCTTTGCGAG
Progesterone receptor (PgR)	CCCGCCGTCGTAACCTTTGG	GTGCTATCCTGCCTCTCAATC
Amphiregulin (AREG)	GTATTTTCACCTTCCGCTCTGTTTTCGCTGGCTATATTGTCGATTCA	
Growth regulation in breast cancer 1 (GREB1)	GAGGATGTGGAGTGGAGACC	CAGTACCTCAAAGACCTCGGC
Forkhead box M1 (FOXM1)	AGCGAGACCCATCAAAGTGG	GGTCTTGGGGTGGGAGATTG
Cell division cycle 25A (CDC25A)	CAAGGGTGCAGTGAACCTTGC	ACAACAATGACACGCTTGCC
Cell division cycle 25B (CDC25B)	CTACTGCTGTGAACCCTGGG	CAACAAAACGCTCCACCTG
Cyclin B1 (CCNB1)	TCTGGATAATGGTGAATGGACA	CGATGTGGCATACTTGTTCTTG
Centromere protein A (CENPA)	ACATGCAGGCCGAGTTACTC	AGAGTCCCCGGTATCATCCC
Polo-like kinase 1 (PLK1)	CTCAACACGCCTCATCCTC	GTGCTCGCTCATGTAATTGC
Cyclin-dependent kinase inhibitor 1A (CDKN1A/p21)	CTGTCTTGTACCCTTGTGCC	GGTAGAAATCTGTCATGCTGG
Endothelial PAS domain protein 1 (EPAS1/HIF2 α)	GCGCTAGACTCCGAGAACAT	TGGCCACTTACTACCTGACCCTT
Vascular endothelial growth factor A (VEGFA)	AGGAGGAGGGCAGAATCATCA	CTCGATTGGATGGCAGTAGCT
Lactate dehydrogenase A (LDHA)	GGCCTGTGCCATCAGTATCT	GCCGTGATAATGACCAGCTT
DNA damage-inducible transcript 4 (DDIT4/REDD1)	AGGAAGCTCATTGAGTTGTG	GGTACATGCTACACACACAT
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	GGGCATCTGGGCTACACTG	GGGCATCTGGGCTACACTG
TATA box binding protein (TBP)	TGCACAGGAGCCAAGAGTGAA	CACATCACAGCTCCCCACCA

2.5. Proliferation Assay

A total of 20,000 cells per well were plated in 24-well multiwell plates. Then, cells were deprived of steroids and serum for 72 h. Cells were treated for 6 days with doses of apigenin ranging from 10^{-8} M to 10^{-5} M or with doses of zearalenone ranging from 10^{-11} M to 10^{-6} M, with or without 10^{-9} M E₂, with retreatment on day 3. After treatment, cells were trypsinized, and the cell number was determined with a TC10 Automated Cell Counter (Bio-Rad).

2.6. Cell Cycle Analysis

A total of 1,000,000 cells per dish were plated in 10 cm dishes. Then, cells were deprived of steroids and serum for 72 h. Cells were treated with solvent as a negative control, 10^{-9} M E2 as a positive control, 10^{-5} M apigenin or 10^{-8} M zearalenone for 72 h. After treatment, cells were trypsinized and fixed with 70% ethanol before being stained with propidium iodide (Sigma) in the presence of RNase A. The cell cycle was analyzed with a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

2.7. Apoptosis Analysis

MCF-7 cells (4000 cells/well) were plated in 96-well plates. After 72 h of serum and steroid deprivation, the cells were treated for 72 h with solvent as a control, 10^{-9} M E2, 10^{-5} M apigenin or 10^{-8} M zearalenone. Terminal deoxynucleotidyl dUTP nick end labeling (TUNEL) staining was assessed with an In Situ Cell Death Detection Kit, Fluorescein (Roche) according to the manufacturer's instructions. The fluorescence intensity and percentage of TUNEL-positive cells were determined with an ArrayScan VTI system (Thermo Fisher Scientific, Waltham, MA, USA) on the ImPACcell platform (Rennes, France).

2.8. Statistical Analysis

One-way analysis of variance (ANOVA) with Dunnett's post hoc test comparing the control treatment to the other treatments was performed with GraphPad Prism software (version 5, GraphPad software, San Diego, CA, USA). Differences were considered significant at a p -value < 0.05 .

2.9. Transcriptomic Analysis

Cells were treated for 24 h with solvent as a control, 10^{-9} M E2, 10^{-5} M apigenin or 10^{-8} M zearalenone RNA extraction, control quality, reverse transcription, labeling and spotting were performed as described in [20]. Sample hybridization, microarray scanning and data extraction were performed by the GeT-Biopuces Platform in Toulouse, France.

2.10. Microarray Data Analysis and Gene Filtration

Data analysis was performed using the Annotation, Mapping, Expression and Network (AMEN) suite of tools [21]. Briefly, probes showing a signal higher than a given background cutoff (corresponding to the overall median of the normalized dataset, 5.20) and at least a 1.5-fold change between the control and treatment signals were selected. To define a set of 3560 transcripts displaying significant statistical changes across comparisons, the linear models for microarray data (LIMMA) package was used (F -value adjusted with the false discovery rate method, $p \leq 0.05$) [22]. The resulting probes were then partitioned into 6 expression clusters (termed C1-C6) using the hierarchical classification on principal component (HCPC) function implemented in the FactoMineR package [23].

2.11. Functional Data Mining

The enrichment analysis module implemented in the AMEN suite of tools [21] was used to identify biological processes significantly associated with each expression pattern by calculating Fisher's exact probability using the Gaussian hypergeometric function (FDR-adjusted p -value ≤ 0.01 , number of probes in a given group associated with a given annotation term ≥ 5).

2.12. Regulatory Network Analysis

Protein-gene regulation data were downloaded from the Transcription Factor Encyclopedia database [24]. A network representation showing all known protein-gene interactions between transcripts differentially expressed in the current project was drawn using AMEN.

3. Results

3.1. ER Is Transactivated by Zearalenone and Apigenin

To test optimal concentrations of zearalenone and apigenin, we first tested the transactivation of ERs in response to different concentrations of these compounds in ER-positive MCF-7 breast cancer cells with a luciferase reporter assay. Cells were transfected with a vector coding for luciferase under the control of an ER-responsive element (ERE) and were treated with various doses of zearalenone (Figure 1A) or apigenin (Figure 1B). Both compounds were able to significantly transactivate ERs at

concentrations starting at 10^{-9} M zearalenone ($p < 0.01$) and 10^{-6} M apigenin ($p < 0.01$), as shown by the increase in luciferase activity. At 10^{-5} M apigenin, luciferase activity reached the same level observed for treatment with 10^{-9} M E2. The maximal activation with zearalenone was observed at 10^{-8} M. To examine the time-dependent activation of ERs, transfected cells were treated with 10^{-9} M E2, 10^{-8} M zearalenone or 10^{-5} M apigenin for 1 h, 3 h, 6 h, 16 h and 24 h (Figure 1C). In the presence of E2 and zearalenone, the activation profile of the luciferase reporter gene was similar. Both E2 and zearalenone stimulated luciferase activity after 3 h of treatment, whereas apigenin induced substantial luciferase activity after 16 h of treatment. Nevertheless, all three compounds similarly stimulated luciferase activity at 24 h, which was therefore used as the treatment time for the next experiments.

To verify that the observed luciferase activity was ER α -dependent, cells were treated with 10^{-9} M E2, 10^{-8} M zearalenone or 10^{-5} M apigenin with or without 10^{-6} M of the antiestrogen ICI_{182,780} (Figure 1D). For E₂, apigenin and zearalenone treatment, the luciferase activity was clearly reduced with ICI_{182,780} compared with that without ICI_{182,780}, thus validating the transactivation of ERs by these molecules.

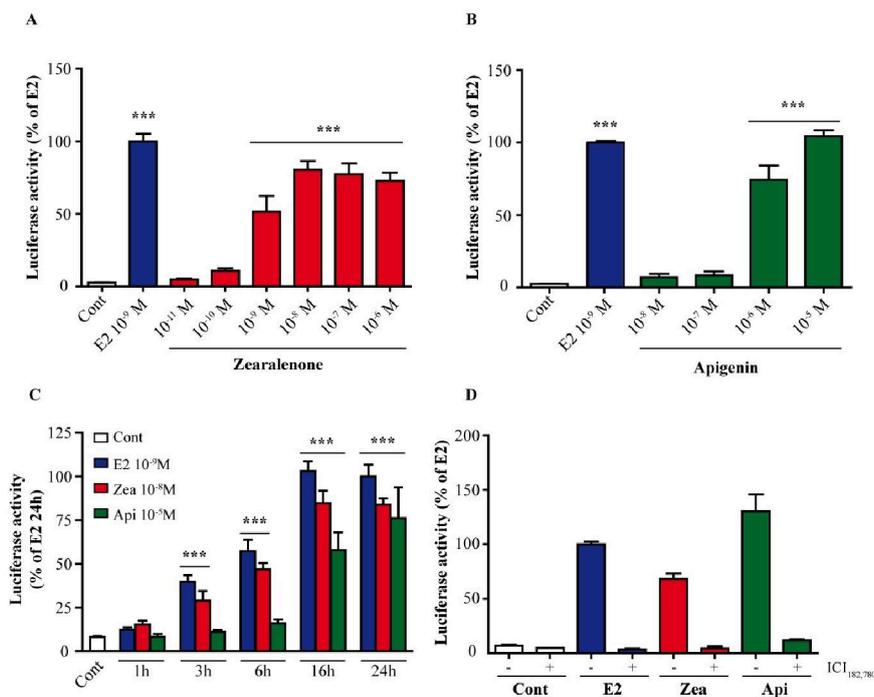


Figure 1. Effect of zearalenone and apigenin on estrogen receptor (ER) activation. MCF-7 cells were transfected with an estrogen-responsive element-thymidine kinase (ERE-TK)-luciferase reporter plasmid and a cytomegalo virus (CMV)- β galactosidase plasmid as a control for transfection efficiency. Then, cells were treated with solvent as a negative control (white), 10^{-9} M E2 as a positive control (blue) or various doses of zearalenone (red) (A) or apigenin (green) (B) for 24 h. The results are expressed as the percentage of luciferase activity attained with E2 treatment and are the means \pm standard error of the mean (SEM) of three to four independent experiments. Cells were treated with solvent as a negative control (white), 10^{-9} M E2 as a positive control (blue), 10^{-8} M zearalenone (red) or 10^{-5} M apigenin (green) for 1 h, 3 h, 6 h, 16 h and 24 h (C). The results are expressed as the percentage of luciferase activity attained with E2 treatment at 24 h and are the means \pm SEM of three independent experiments. (D) To confirm the estrogenic effects of apigenin and zearalenone, transfected cells were cotreated with 10^{-6} M ICI_{182,780} and either 10^{-9} M E2 (blue) or 10^{-8} M zearalenone (red) or 10^{-5} M apigenin (green). *** indicates a p -value < 0.001 by one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test for comparison of the control treatment with the other treatments.

3.2. Zearalenone and Apigenin Are Able to Induce the Recruitment of ER α DNA-Binding at Chromatin Sites

To test whether the observed differential kinetics between zearalenone and apigenin were due to a change in the recruitment of ER α to chromatin, we performed ChIP-PCR analysis on 4 distinct sites after 1 h of treatment with 10^{-9} M E2, 10^{-8} M zearalenone or 10^{-5} M apigenin (Figure 2). The binding sites studied were the proximal promoter of GREB1, which contains a consensus ERE sequence (Figure 2A), the superenhancer of CUEDC1, which also contains an ERE sequence but is localized in the core of the gene [25] (Figure 2B), and the two binding sites in the XBP1 enhancer (Figure 2C). The first contains 3 half-ERE binding sites, while the second contains one AP-1 and one SP-1 binding site. For all binding sites studied, both zearalenone and apigenin were able to significantly induce the recruitment of ER α as E2 did. This result indicates that the differential activation of ER target genes by apigenin versus that induced by E2 and zearalenone likely occurs at the transcriptional level and not at ER binding sites.

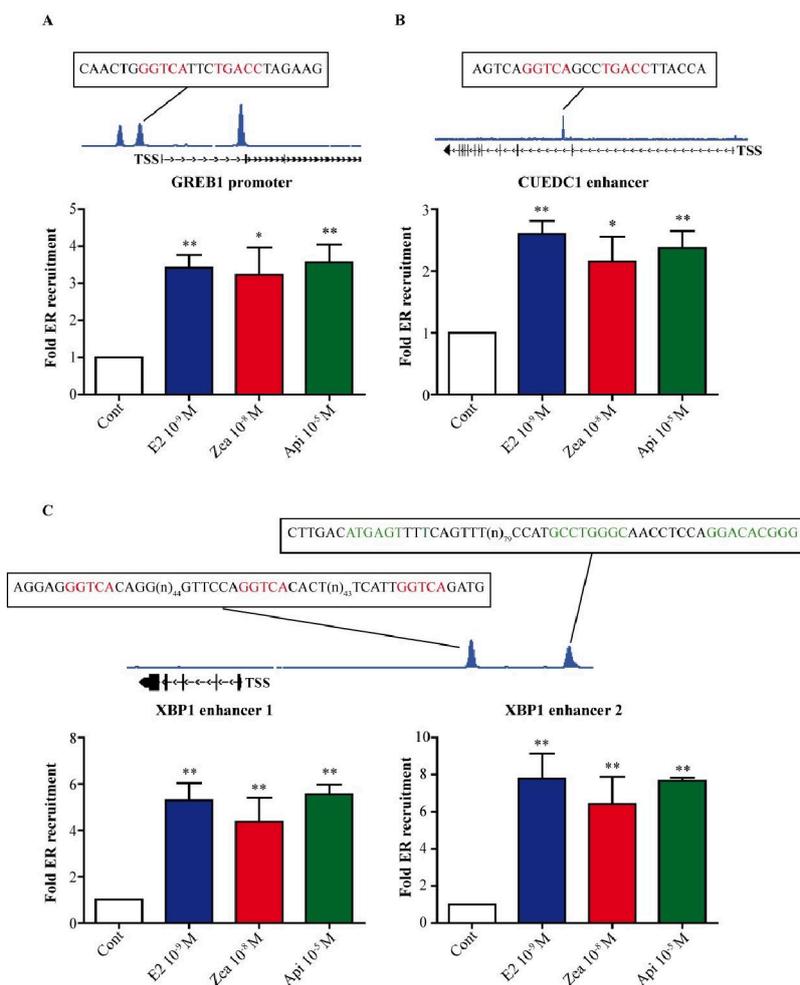


Figure 2. ER α recruitment to chromatin at distinct ER binding sites in response to E2, zearalenone and apigenin. MCF-7 cells were treated with solvent (white) as a negative control, 10^{-9} M E2 (blue) as a positive control, 10^{-8} M zearalenone (red) or 10^{-5} M apigenin (green) for 1 h. The recruitment of ER α to the GREB1 promoter (A), CUE domain containing 1 (CUEDC1) enhancer (B) and the two enhancers of x-box protein 1 (XBP1) (C) was assessed by chromatin immunoprecipitation followed by real-time PCR. For each binding site tested, the DNA sequence is indicated. The results are expressed in fold recruitment compared to control and are the means of four independent experiments. * indicates a p -value < 0.05 and ** indicates a p -value < 0.01 by one-way ANOVA followed by Dunnett's post hoc test for comparison of the control treatment with the other treatments.

3.3. Induction of E2-Dependent Genes Is Different between Zearalenone and Apigenin

As zearalenone and apigenin activated ERs, we tested the potential effect of these molecules on the expression of endogenous E2-dependent genes, such as the chemokine CXCL12, the progesterone receptor (PgR), amphiregulin (AREG) and growth regulation in breast cancer 1 (GREB1) (Figure 3). MCF-7 cells were treated for 24 h with 10^{-9} M E2 as a positive control and with various doses of zearalenone (Figure 3, left) or apigenin (Figure 3, right). Although both compounds induced E2-dependent gene expression, marked dose-response differences were observed between zearalenone and apigenin. Indeed, zearalenone treatment resulted in an increase in gene expression starting at 10^{-10} M; this increase became significant compared to that the gene expression in the control cells at 10^{-9} M for PgR, AREG and GREB1 and at 10^{-8} M for CXCL12. Only the highest tested dose of apigenin induced a significant change in gene expression that reached the same level as produced by E2 treatment for any tested gene except for PgR, for which the effect reached only 60% of that induced by E2. Furthermore, the effect of zearalenone and apigenin on the expression of CXCL12 gene was examined in T47D (Figure S1A). Interestingly, zearalenone was more potent than apigenin in inducing CXCL12 gene expression.

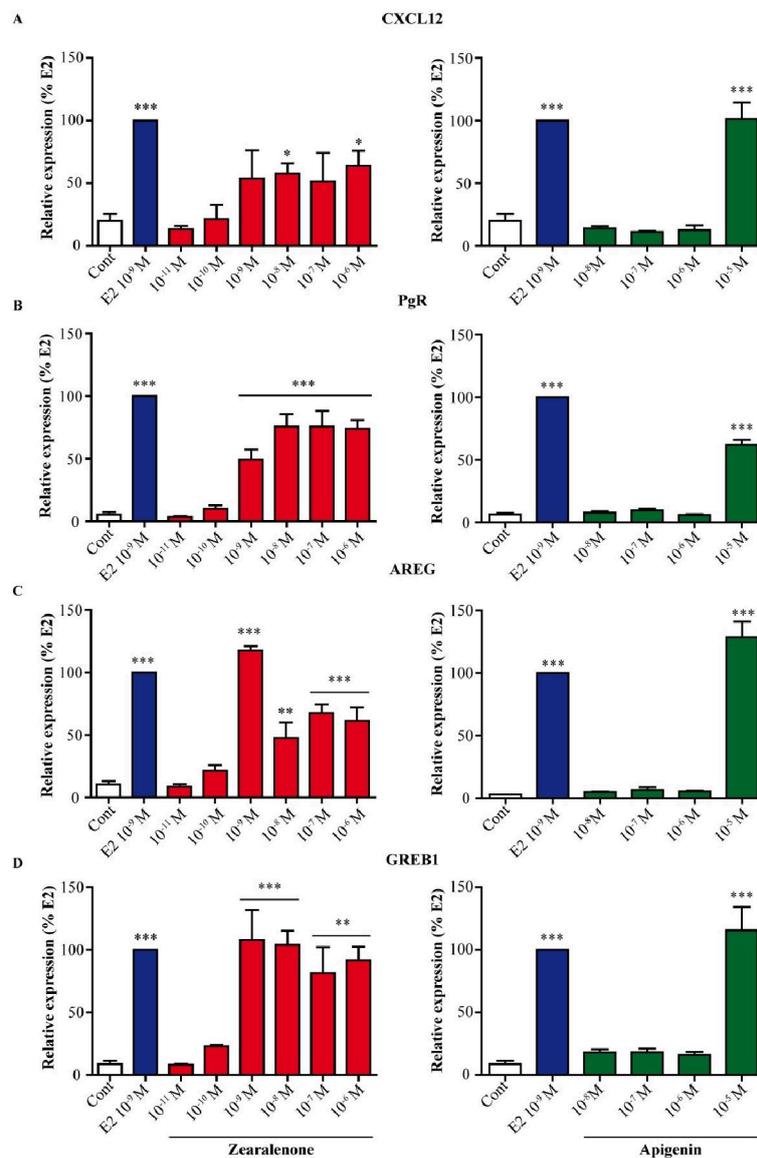


Figure 3. Effect of zearalenone and apigenin on the expression of endogenous E2 target genes. MCF-7 cells were treated with solvent (white) as a negative control, 10^{-9} M E2 (blue) as a positive control or with various doses of zearalenone (red) or apigenin (green) for 24 h. The expression of CXCL12 (A), PgR (B), AREG (C) and GREB1 (D) was assessed by real-time PCR. The expression level of each gene was normalized to the expression levels of the housekeeping genes GAPDH and TBP. The results are expressed as the percentage of the relative expression of transcripts obtained in E2-treated cells and are the means \pm SEM of three independent experiments. * indicates a p -value < 0.05 , ** indicates a p -value < 0.01 , and *** indicates a p -value < 0.001 by one-way ANOVA followed by Dunnett's post hoc test for comparison of the control treatment with the other treatments.

3.4. Zearalenone and Apigenin Have Different ER-Dependent Proliferative Effects on MCF-7 Cells

To characterize the ER-dependent proliferative effects of these two compounds, we first analyzed their impact on MCF-7 cell number (Figure 4). Cells were treated with various doses of zearalenone (Figure 4A, solid line) or apigenin (Figure 4B, solid line) for 6 days. Zearalenone induced proliferation starting at 10^{-10} M, and the effect reached a plateau at approximately 80% of the effect of 10^{-9} M E2. Apigenin induced a slight increase in cell number starting at 10^{-6} M and reaching a maximum at 10^{-5} M, and the cell number represented only 50% of the cell number observed after treatment with 10^{-9} M E2. In a second experiment, cells were cotreated with a single concentration of E2 (10^{-9} M) and various doses of zearalenone (Figure 4A, dotted line) or apigenin (Figure 4B, dotted line). Under these conditions, zearalenone had no effect on the proliferation induced by 10^{-9} M E2. In contrast, apigenin showed an antagonistic effect, with a reduction in cell number to approximately 65% of that with E2 treatment only, at 10^{-5} M. Since cell number is the result of cell proliferation and cell death, we assessed the effect of treatment with 10^{-8} M zearalenone and 10^{-5} M apigenin on the cell cycle (Figure 4C) and apoptosis (Figure 4D). The cell cycle was analyzed by flow cytometry after propidium iodide staining, and the percentage of cells in each cell cycle phase was determined. As expected, E2 induced the entry of cells into the cell cycle, with a significant increase in the percentage of cells in the S and G2/M phases compared to that of control cells. Zearalenone and apigenin also promoted the entry of cells into the cell cycle by significantly increasing the percentage of cells in the S phase. However, the percentage of cells in the G2/M phase was not significantly increased compared to that of the control cells (Figure 4C). Apoptosis was determined by a TUNEL assay (Figure 4D). As described in numerous studies, E2 treatment significantly reduced the percentage of TUNEL-positive cells. Zearalenone and apigenin also reduced the percentage of TUNEL-positive cells, but this reduction was not significant compared to the percentages of TUNEL-positive control cells or E2-treated cells. In parallel, we examined the effects of zearalenone and apigenin alone and in combination with estradiol on the proliferation of T47D ER-positive breast cancer cells. Using this model, we confirmed the partial antagonistic effect of apigenin on the E2-dependent proliferation of T47D, while zearalenone alone or in combination with estradiol showed a full agonistic effect (Figure S2).

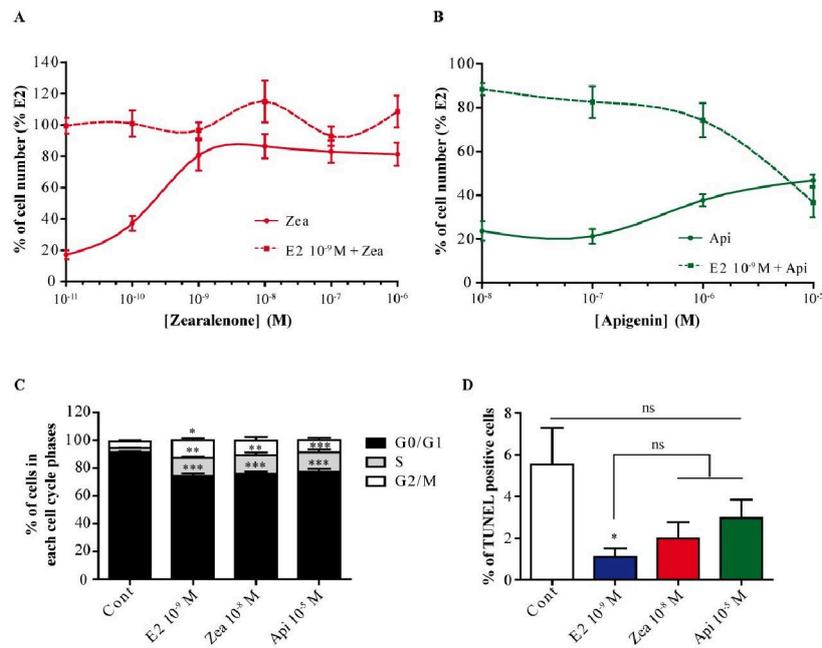


Figure 4. Effect of zearalenone and apigenin on MCF-7 proliferation. MCF-7 cells were treated with various doses of zearalenone (A) or apigenin (B) alone (solid lane) or in combination with 10^{-9} M E2 (dotted line) for 6 days, and the cell number was determined by counting. The results are expressed as the percentage of the cell numbers counted after E2 treatment and are the means \pm SEM of three to four independent experiments. For the cell cycle (C) and apoptosis (D) assays, MCF-7 cells were treated for 3 days with 10^{-9} M E2, 10^{-8} M zearalenone or 10^{-5} M apigenin. The cell cycle was analyzed by flow cytometry after propidium iodide staining. The results are expressed as the percentage of cells in each phase of the cell cycle and are the means \pm SEM of three to four independent experiments. Apoptosis analysis was performed by a terminal deoxynucleotidyl dUTP nick end labeling (TUNEL) assay, and the percentage of apoptotic cells was assessed with an Array Scan VTI. The results are expressed as the percentage of TUNEL-positive cells. ns indicates a non-significant p -value, * indicates a p -value < 0.05 , ** indicates a p -value < 0.01 , and *** indicates a p -value < 0.001 by one-way ANOVA followed by Dunnett's post hoc test for comparison of the control treatment with the other treatments.

3.5. Genome Wide Microarray Analysis

To gain more insight into the gene networks differentially regulated by zearalenone and apigenin and to determine how these compounds may influence the cellular phenotypes, a genome-wide microarray analysis was performed. Cells were treated with solvent as a control, 10^{-9} M E2, 10^{-8} M zearalenone or 10^{-5} M apigenin. The data were deposited in gene expression omnibus (GEO) (GSE1195) and in TOXsIqN (TSP763) [26]. After the statistical filtration analysis as described in the materials and methods section and in Figure 5A, 3560 probes showing a significant differential expression were selected and further classified into six expression clusters (termed C1-6). The C1-3 clusters are associated with genes (1826 probes corresponding to 1510 genes) showing a downregulated expression pattern as compared to the control samples, while C4-C6 for the three tested compounds contain genes (1498 probes, 1476 genes) significantly induced after the exposure to E2, zearalenone and apigenin (Figure 5A and Table S1). Of particular interest was that, at the dose tested, E2, zearalenone and apigenin altered the expression of the same set of genes (Figure 5B,C). Nevertheless, while E2 and zearalenone had a very similar impact on the transcriptional program of MCF-7 cells by affecting genes at similar expression level (as shown in Figure 5B,C), the level of over- or under-expression induced by apigenin is always weaker (C3 and C6) or stronger (C2, C4 or C5) than those induced by E2. To deepen the analysis, we performed a functional analysis by identifying, the major biological processes associated with those different clusters are shown in Figure 5D. Importantly, we found that

underexpressed gene clusters (C1) to be significantly enriched in genes involved in cell differentiation and cell communication, while overexpressed genes clusters (C4 and C6) were mainly linked to cell cycle and cell growth.

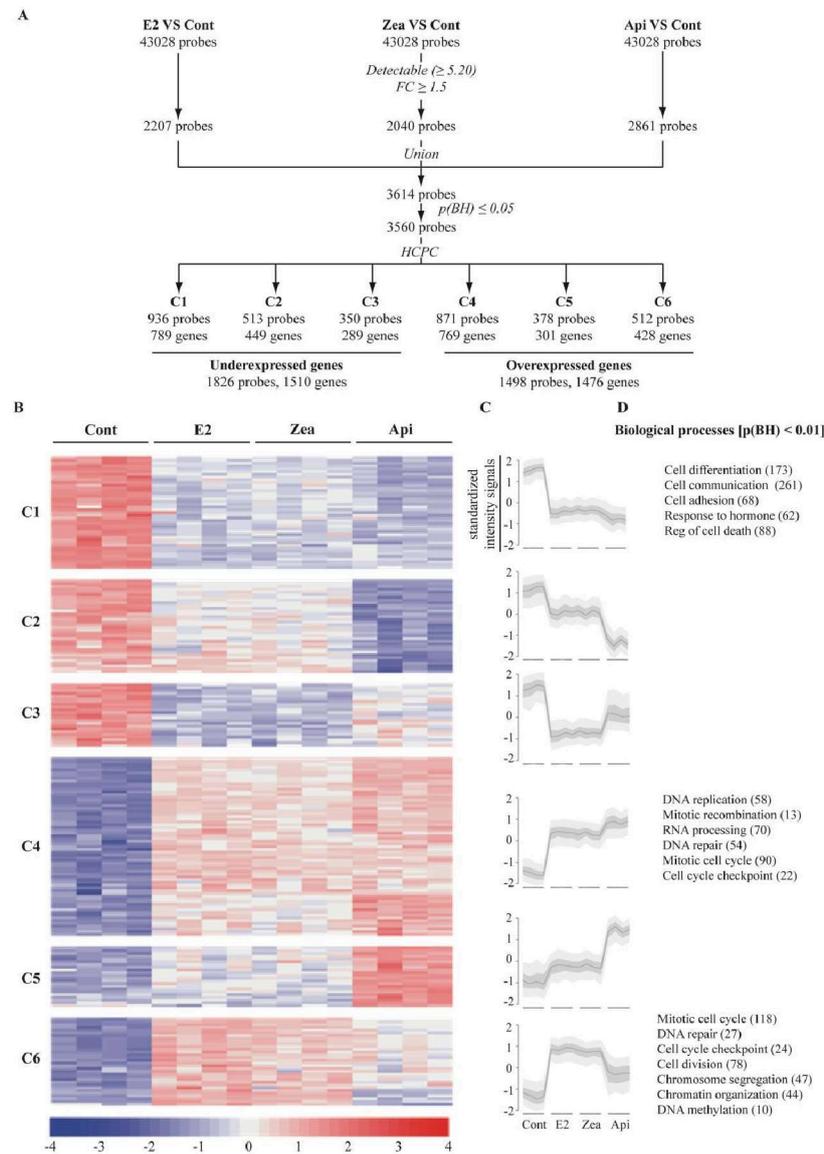


Figure 5. Transcriptomic analysis, selection and clustering of differentially expressed genes. MCF-7 cells were treated with solvent (Cont), 10^{-9} M E2, 10^{-8} M zearalenone (Zea) or 10^{-5} M apigenin (Api) for 24 h. Total RNA was extracted, reverse transcribed, labeled and spotted onto a DNA chip. (A) To select differentially expressed genes, each treatment was compared to the control, and all probes with both an intensity signal above the overall median and a fold change ≥ 1.5 were chosen. Then, the probes were combined and submitted to a LIMMA test; only probes with a p -value < 0.05 were selected, resulting in a total of 1510 underexpressed genes and 1498 overexpressed genes. (B) These genes were clustered into 6 clusters depending on their expression patterns. (C) Intensity signals were standardized, and the expression profile of each cluster is presented. Major biological processes significantly associated (p -value < 0.01) with the different clusters are noted (D). The number of differentially expressed genes corresponding to each gene ontology (GO) term are indicated in parentheses.

3.6. Regulatory Network Analysis Revealed

A gene-protein interaction network was built with all the differentially expressed genes (Figure 6 and Table S1), to which ER α was added. This network showed a large network composed of three communities centered on 7 transcription factors. A small community was composed of the ER α (ESR1), glucocorticoid receptor (GR, NR3C1) and androgen receptor (AR) genes, which were globally downregulated. The second community was centered on forkhead box M1 (FOXO1), which is a master regulator of several genes involved in the cell cycle, and on E2F transcription factor 1 (E2F1). These genes were upregulated. The third community was linked to hypoxia inducible factor 1 alpha (HIF1 α)/ endothelial PAS domain protein 1 (EPAS1, HIF2 α). These two transcription factors are well-described as key factors in controlling a responsive gene network during hypoxia or as being involved in cell growth arrest or metabolic reprogramming. In this community, half of the genes were upregulated, and half were downregulated. Two other small networks were also defined. One was centered on the transcription factor forkhead box O1 (FOXO1), which was downregulated. The other was centered on retinoid X receptor alpha (RXRA), which was also downregulated.

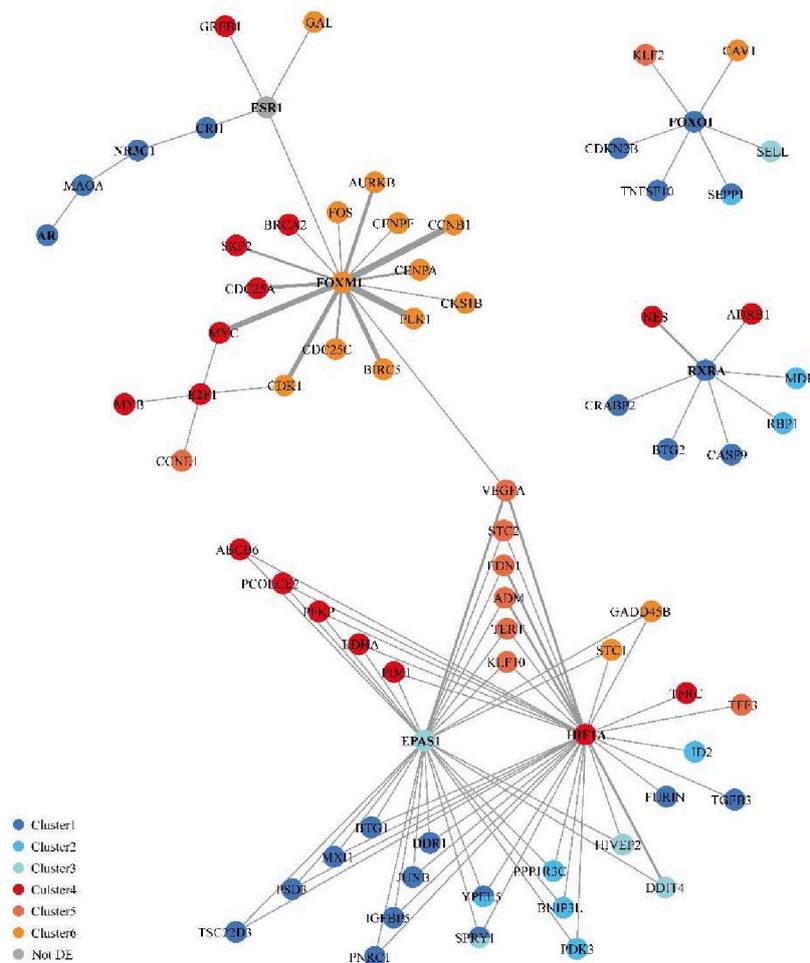


Figure 6. Gene regulation network built from the transcript profiling data and regulation data.

Each gene/protein is represented by a node color-coded according to the related expression clusters. The edges between the nodes correspond to the protein/DNA interactions, and the thickness of each edge is linked to the number of publications describing this interaction. Transcription factors are indicated in bold.

3.7. Zearalenone and Apigenin alter the Expression of Genes Involved in Cell Cycle and Growth Arrest

In cluster 6 and for the most part in cluster 4, gene ontology (GO) terms associated with the cell cycle were significantly ($p < 0.01$) enriched. Notably, the transcription factor FOXM1, which was differentially expressed, controls the expression of numerous genes involved in cell cycle progression. Thus, we first validated our transcriptomic data for several genes involved in cell cycle progression, such as FOXM1 (Figure 7A), cell division cycle 25A (CDC25A) (Figure 7B), cell division cycle 25B (CDC25B) (Figure 7C), cyclin B1 (CCNB1) (Figure 7D), centromere protein A (CENPA) (Figure 7E), polo like kinase 1 (PLK1) (Figure 7F) and cyclin dependent kinase inhibitor 1A CDKN1A (p21^{cip1}) (Figure 7G). Compared to their levels in control cells, genes belonging to cluster 4 (FOXM1, CDC25B, CCNB1, CENPA and PLK1) were significantly upregulated ($p < 0.01$) by 10^{-9} M E2 and 10^{-8} M zearalenone, while apigenin 10^{-5} M did not affect the expression of these genes. CDC25A belonged to cluster 6 and was slightly but not significantly upregulated by E2 and zearalenone, while apigenin upregulated CDC25A expression significantly ($p < 0.01$). Finally, CDKN1A, which is involved in cell cycle arrest, was significantly downregulated ($p < 0.01$) by E2 and zearalenone and was slightly but not significantly downregulated by apigenin.

Another community in the network was centered on the transcription factors HIF1 α and EPAS1/HIF2 α . These transcription factors are involved in the response to hypoxia not only by inducing genes such as vascular endothelial growth factor A (VEGFA) and lactate dehydrogenase A (LDHA) but also by regulating growth arrest genes such as DNA damage inducible transcript 4 (DDIT4). As shown in Figure 8A, E2 and zearalenone strongly downregulated the expression of the transcription factor EPAS1/HIF2 α (by 4 times; $p < 0.001$) while apigenin resulted in a reduction of only 2 times ($p < 0.05$). Of particular interest was that DDIT4, which is an inhibitor of mammalian target of rapamycin (mTOR) and is involved in cell growth, was significantly downregulated by E2 and zearalenone ($p < 0.001$) but not by apigenin (Figure 8B). On the other hand, the expression of VEGFA, which was significantly induced by apigenin ($p < 0.05$), was not modified by E2 or zearalenone (Figure 8C), while the expression of LDHA was significantly induced by E2 and zearalenone ($p < 0.05$) but not by apigenin (Figure 8D). Differential regulation of DDIT4 gene expression was also confirmed in T47D cells (Figure S1C).

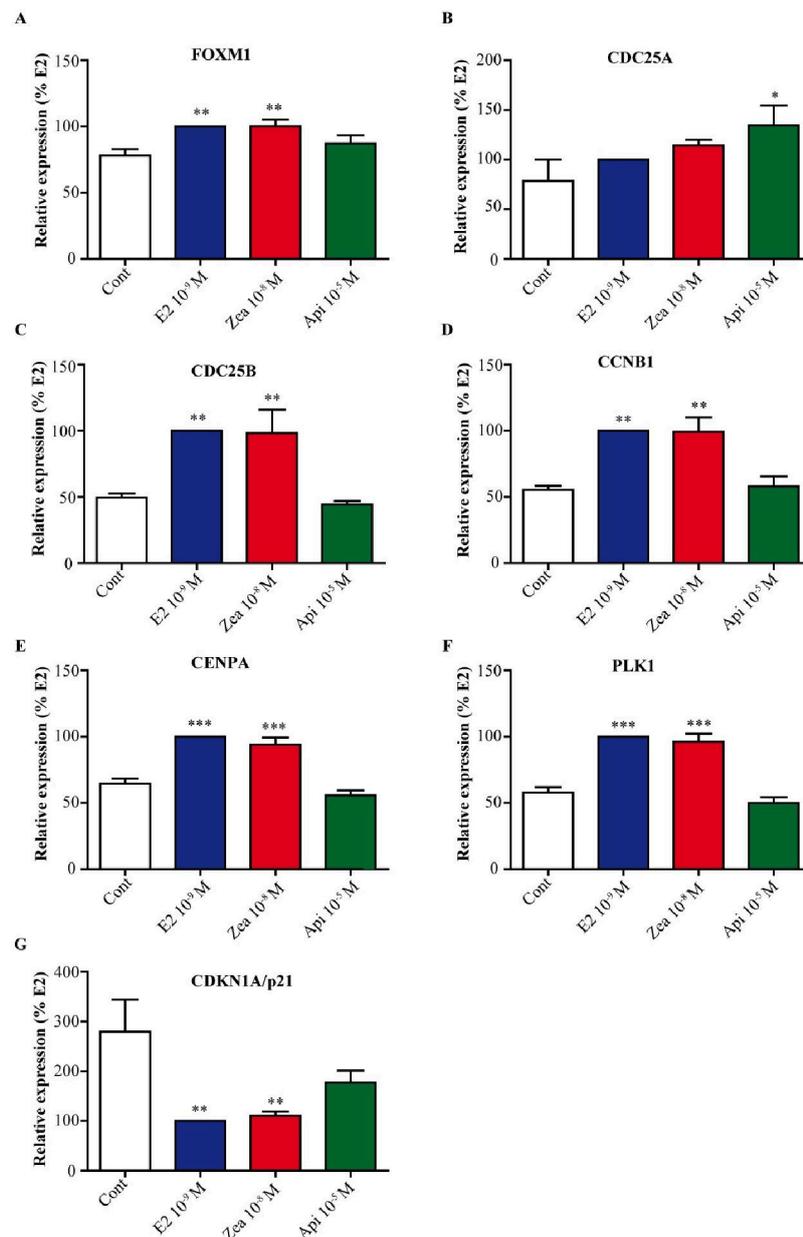


Figure 7. Validation of cell cycle-associated genes linked to forkhead box M1 (FOXMI). MCF-7 cells were treated with solvent (white) as a negative control, 10⁻⁹ M E2 (blue) as a positive control, 10⁻⁸ M zearalenone (red) or 10⁻⁵ M apigenin (green) for 24 h. The expression of FOXMI (A), cell division cycle 25A (CDC25A) (B), cell division cycle 25B (CDC25B) (C), cyclin B1 (CCNB1) (D), centromere protein A (CENPA) (E), polo like kinase 1 (PLK1) (F) and cyclin dependent kinase inhibitor 1A (CDKN1A/p21) (G) was assessed by real-time PCR. The expression level of each gene was normalized to the expression levels of the housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and TATA box binding protein (TBP). The results are expressed as the percentage of relative expression of transcripts obtained in E2-treated cells and are the means \pm SEM of six independent experiments. * indicates a *p*-value < 0.05, ** indicates a *p*-value < 0.01 and *** indicates a *p*-value < 0.001 by one-way ANOVA followed by Dunnett's post hoc test for comparison of the control treatment with the other treatments.

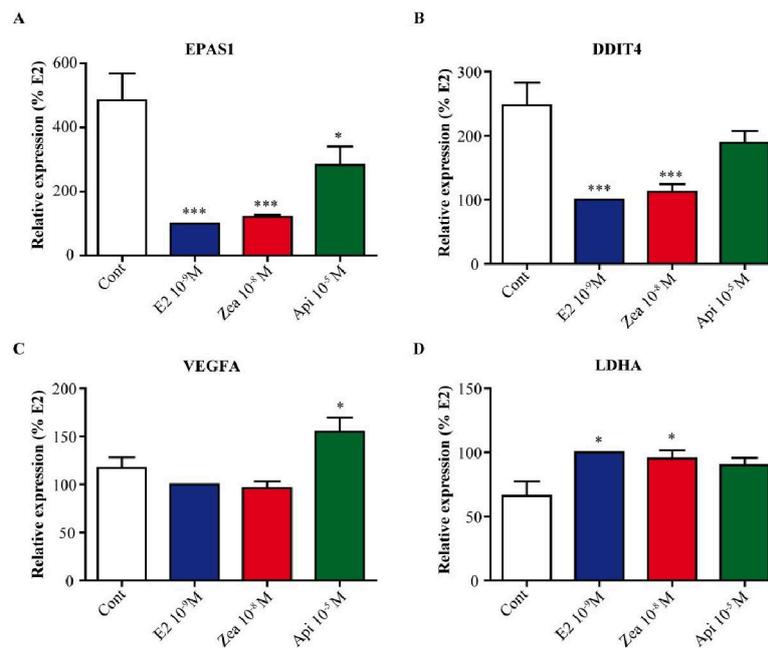


Figure 8. Validation of metabolism and growth arrest genes linked to HIF1 α and EPAS1/HIF2 α . MCF-7 cells were treated with solvent (white) as a negative control, 10⁻⁹ M E2 (blue) as a positive control, 10⁻⁸ M zearalenone (red) or 10⁻⁵ M apigenin (green) for 24 h. The expression of EPAS1/HIF2 α (A), DNA damage inducible transcript 4 (DDIT4) (B), vascular endothelial growth factor A (VEGFA) (C) and lactate dehydrogenase A (LDHA) (D) was assessed by real-time PCR. The expression level of each gene was normalized to the expression levels of the housekeeping genes GAPDH and TBP. The results are expressed as the percentage of the relative expression of transcripts obtained in E2-treated cells and are the means \pm SEM of six independent experiments. * indicates a p -value < 0.05 and *** indicates a p -value < 0.001 by one-way ANOVA followed by Dunnett's post hoc test for comparison of the control treatment with the other treatments.

3.8. Zearalenone and Apigenin Alter the Expression of Genes Linked to Cancer, Nucleoli and Apoptosis

Furthermore, the expression of various genes in the different clusters described above was validated by real-time PCR (Figure 9). Thus, in connection with breast cancer, we examined the expression of genes encoding oncogenes that promote tumorigenesis or those associated with a poor prognosis, such as ADAM metalloproteinase with thrombospondin type 1 motif 4 (ADAMTS4), CAMP-dependent protein kinase inhibitor beta (PKIB), peripheral myelin protein 22 (PMP22), and inhibitor of differentiation 1 (ID1) [27–30]. Interestingly, ADAMTS4 and PKIB were significantly upregulated by E2 and zearalenone ($p < 0.001$) but were much less upregulated by apigenin ($p < 0.05$ and $p < 0.01$, respectively) (Figure 9A,B). We confirmed this result in T47D cells, where the expression of ADAMTS4 gene was markedly induced by E2 and zearalenone but not by apigenin (Figure S1B). On the other hand, the PMP22 and ID1 genes were significantly downregulated by E2 and zearalenone, but ID1 gene expression was more strongly downregulated by apigenin ($p < 0.001$) (Figure 9C,D). RBM24 (RNA binding motif 24) (Figure 9E) has not been described in breast cancer, but this gene was characterized to suppress nasopharyngeal carcinoma progression [31]. In addition, RBM24 has an essential role in mediating the proper expression of p53 [32]. Our work shows for the first time that the RBM24 gene is significantly upregulated by E2, zearalenone and apigenin ($p < 0.001$), suggesting that RBM24 may be a new ER target gene in breast cancer. In cluster 5, AEN (apoptosis-enhancing nuclease) (Figure 9F), which is associated with p53-mediated apoptosis, was significantly upregulated by zearalenone and apigenin ($p < 0.01$ and $p < 0.001$, respectively) suggesting a potential stress induced by these two compounds. Moreover, NOLC1 (nucleolar and coiled-body phosphoprotein 1) (Figure 9G) as well as BOP1 (block of proliferation 1) (Figure 9H) are involved in RNA processing in the nucleoli.

Thus, it could be interesting to consider that apigenin upregulated these two genes more strongly than E2 and zearalenone did.

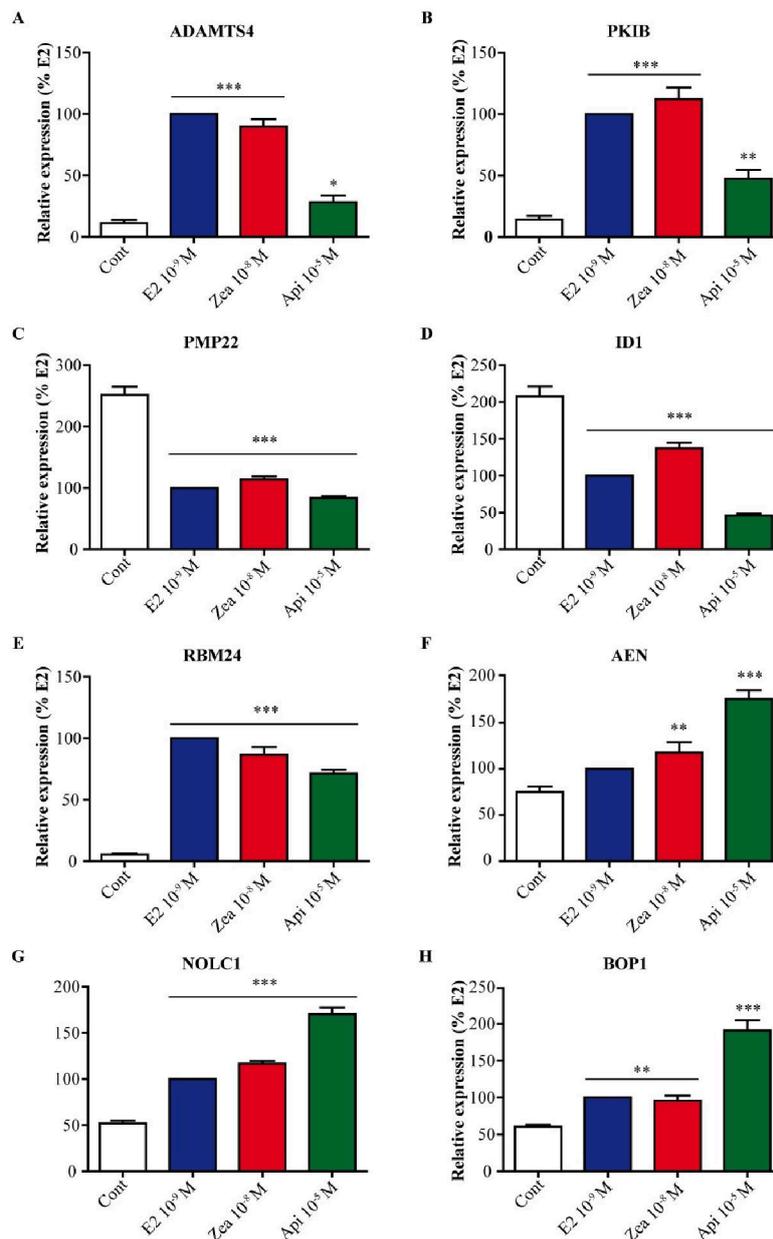


Figure 9. Validation of genes linked to cancer, nucleoli and apoptosis. MCF-7 cells were treated with solvent (white) as a negative control, 10⁻⁹ M E2 (blue) as a positive control, 10⁻⁸ M zearalenone (red) or 10⁻⁵ M apigenin (green) for 24 h. The expression of ADAM metalloproteinase with thrombospondin type 1 motif 4 (ADAMTS4) (A), CAMP-dependent protein kinase inhibitor beta (PKIB) (B), peripheral myelin protein 22 (PMP22) (C), inhibitor of differentiation 1 (ID1) (D), RNA binding motif 24 (RBM24) (E), apoptosis-enhancing nuclease (AEN) (F), nucleolar and coiled-body phosphoprotein 1 (NOLC1) (G) and block of proliferation 1 (BOP1) (H) was assessed by real-time PCR. The expression level of each gene was normalized to the expression levels of the housekeeping genes GAPDH and TBP. The results are expressed as the percentage of the relative expression of transcripts obtained in E2-treated cells and are the means ± SEM of six independent experiments. * indicates a *p*-value < 0.05, ** indicates a *p*-value < 0.01 and *** indicates a *p*-value < 0.001 by one-way ANOVA followed by Dunnett's post hoc test for comparison of the control treatment with the other treatments.

4. Discussion

Breast cancer is a major public health concern with an estimated economic cost of €15 billion in the European Union [33]. The etiology of breast cancer is complex, with multiple causes (genetic, environmental aggression, etc.) and large heterogeneity. Dietary is an important part of our environment. It is now well established that diet acts on our health. For instance, a recent study showed that organic food consumption reduced cancer risk [34], even if this study needs to be confirmed. Zearalenone and apigenin are found in the diet and, consequently, may impact the etiology of breast cancer; zearalenone is an exogenous toxin, and apigenin is an endogenous compound in numerous plant foods. Using different approaches, we and others showed that these two compounds are able to bind and activate ERs [5,35–38]. Recently, we showed that zearalenone and apigenin have differential effects on ER α -mediated proliferation and differentiation [5]. In this work, we studied the molecular mechanisms sustaining the activity of apigenin and zearalenone on ER-positive MCF-7 breast cancer cells proliferation.

Our results confirmed that zearalenone and apigenin activate ER α on the ERE-controlled luciferase reporter gene, although a difference in the kinetics of luciferase activity was observed. ChIP experiments showed no major differences in ER α recruitment to various ER binding sites when ER α was activated by zearalenone or apigenin. However, zearalenone elicited a full and powerful proliferative effect on breast cancer cells in both the absence and presence of E2, while apigenin showed a partial and weak proliferative effect in the absence of E2 and an antagonistic effect in the presence of E2. We showed these effects in both ER-positive breast cancer cell lines, MCF-7 and T47D. Taken together, these results suggest that apigenin is a partial agonist, while zearalenone is a full ER α agonist.

ER α has two transactivation domains; AF-1 is located in the N-terminus and is ligand-independent, whereas AF-2 is in the C-terminus in the ligand-binding domain (LBD). AF-2 contains a flexible region corresponding to the helix 12 which is important for the recruitment of co-factor. The binding of E2 to ER α gives rise to a conformational change in ER α which allows the active positioning of the helix 12 in AF2 transactivation and a synergistic effect between AF-1 and AF-2 for the recruitment of coactivators such as SRC-1 [39]. ER structure-function studies have reported that certain estrogen-like compounds allow the establishment of a conformation distinct from that induced by E2. This remarkable property for these molecules would offer promising therapeutic prospects in E2-associated diseases. In a recent work using different AF-2 mutants of ER α , Arao et al. showed that zearalenone and E2 induced a conformation of ER α that fully activated ER α even with the mutation of the static region of AF-2. Conversely, apigenin was not able to induce ER α bearing a mutation in AF-2, suggesting that the conformation of the LBD was not optimal [36,40]. Moreover, docking experiments of zearalenone and apigenin to the LBD of ER α showed a real difference in docking between these compounds. Indeed, it was demonstrated that the interaction of ligands with His-524 was necessary for full agonist activity [41]. Zearalenone interacts with His-524, which permits the proper folding of helix 12 and the recruitment of coactivators [38,42]. In contrast, apigenin interacts very weakly or not at all with His-524 [37]. To obtain a global insight into the effect of these compounds on ER-mediated transcription that could also explain their differential effects on cellular fate, a genome-wide microarray analysis of MCF-7 breast cancer cells was performed. Interestingly, this analysis demonstrated that both compounds regulate the expression of ER-target genes in the same direction but at different intensities, which could be explained by the differential interaction of zearalenone and apigenin with the LBD of ER α . As suggested previously, depending on the chromatin context, including the presence of epigenetic marks, the mechanism of binding to an ERE directly or by tethering (via AP-1 and SP-1) and the concentration of cofactors, the intensity of ER target gene regulation could be different [43]. Our transcriptomic analysis revealed two regulatory networks, namely, a hub centered on FOXM1 and ER α and a hub centered on HIF1 α /EPAS1, as also found in a previous work on the glyceollin phytoalexins [20]. This result indicates the existence of common links between these molecules and molecular pathways for the growth and apoptosis of breast cancer cells. Indeed, FOXM1

is a key regulator of several genes involved in cell cycle progression, especially in the G1/S and G2/M transitions [44]. In contrast to zearalenone and E2, apigenin did not enhance the expression of FOXM1, and all tested genes except CDC25A followed the expression pattern of this transcription factor. For instance, the CDC25 family (CDC25A-C) is a new target for the treatment of triple-negative breast cancer [45]. In our study, apigenin did not induce the expression of two members of this family (CDC25B and CDC25C). A cell cycle analysis showed that all treatments induced cell entry into the G1/S phase. In accordance with this observation, CDC25A expression was induced, although only apigenin treatment resulted in significant upregulation. However, treatment with zearalenone or apigenin did not result in a significant increase in the percentage of cells in the G2/M phase compared to that of the control cells. The expression of CDC25B or CCNB1, which are involved in G2/M progression, was in accordance with this observation for apigenin but not for zearalenone, suggesting a possible induction of checkpoint control by zearalenone treatment. This hypothesis is reinforced by the slight increase in the percentage of TUNEL-positive zearalenone-treated cells compared to the percentage of TUNEL-positive E2-treated cells.

The second hub in the regulatory network was centered on the HIF1 α and EPAS1 transcription factors. This family of transcription factors is well-described for their role in adaptation to low-O₂ conditions [46]. HIF1 α was slightly increased by treatment with all compounds tested (Table S1), while EPAS1/HIF2 α were clearly downregulated by E2 and zearalenone treatment and less by apigenin treatment. This result is consistent with those in our previous work and in other studies showing that estrogens upregulate the expression of HIF1 α and downregulate the expression of the EPAS1/HIF2 α genes [20,47,48]. Consequently, the expression of direct HIF target genes such as lactate dehydrogenase A (LDHA) or vascular endothelial growth factor A (VEGFA) was upregulated by E2 and zearalenone or by apigenin, respectively. These genes are involved in the adaptation of cells to hypoxia and to neovascularization, respectively. More importantly, the expression of DDIT4 (also known as REDD1), an inhibitor of mTOR [49], was significantly downregulated by E2 and zearalenone but not by apigenin. mTOR is a downstream effector of the PI3K/AKT pathway, which regulates protein synthesis by promoting the phosphorylation of p70S6K.

In parallel with the direct effect of treatment on gene expression, the impact of apigenin and zearalenone on intracellular pathways might explain their proliferative or antiproliferative activity. For example, the inhibition of the PI3K/AKT/mTOR pathway by apigenin was described in several cancers, including prostate cancer [50], lung cancer [51] and hepatocellular carcinoma [52]. Another major pathway involved in the proliferation of breast cancer cells is the MAPK pathway, which is involved particularly via a rapid and transient interaction between ER α and the intracellular SRC proto-oncogene, non-receptor tyrosine kinase (c-Src/Src) [16]. Using a proximity ligation assay, we observed a slight increase in ER α /Src interaction in response to E2 and zearalenone treatment, while apigenin treatment weakly but not significantly repressed this interaction (Figure S3). The decrease in this interaction was accompanied by a weak decrease in the phosphorylation of Src after 30 min (Figure S4A) and an increase in p53 phosphorylation after 24 hours of apigenin treatment compared to E2 (Figure S4B) observed with the proteome profiler kit. These results on the regulation of gene expression and the modulation of intracellular pathways suggest that apigenin induces a senescence phenotype characterized by the sustained expression of growth arrest genes and by the induction of the p53 pathway, as confirmed by the induction of AEN or RBM24.

5. Conclusions

In this study, we sought to decipher molecular mechanisms sustaining the estrogenic activity of zearalenone and apigenin in an ER-positive breast cancer cell model, and to better understand how these compounds could differentially affect cell fate. This study, like others before, confirmed the selective estrogen receptor modulator (SERM) potential of apigenin in ER-positive breast cancer and did not contest its potential health benefit effect. However, a drawback concerning the use of apigenin is its low bioavailability. It will be interesting to increase this bioavailability by chemical

functionalization or galenic approaches. For instance, in one study, the use of a carbon nanopowder succeeded in increasing the bioavailability of apigenin [53]. In contrast, zearalenone appears to be a true endocrine-disrupting compound that may contribute to tumorigenesis even at very low doses, as was suggested in a recent review [54].

Supplementary Materials: The following are available online at <http://www.mdpi.com/2072-6643/11/2/237/s1>, Table S1: All the genes differentially expressed found in the transcriptomic analysis were presented with their names, their gene ontologies, and their regulations, Figure S1: T47D cells were treated with solvent (white) as a negative control, 10^{-9} M E2 (blue) as a positive control or with 10^{-8} M zearalenone (Zea, red) or with 10^{-5} M apigenin (Api, green) for 24 h. The expression of CXCL12 (A), ADAMTS4 (B), DDIT4 (C), CDC25B (D) and BOP1 (E) was assessed by real-time PCR. The expression level of each gene was normalized to the expression levels of the housekeeping genes GAPDH and TBP. The results are expressed as the percentage of the relative expression of transcripts obtained in E2-treated cells and are the means \pm SEM of at least three independent experiments, Figure S2: T47D cells were grown for 6 days in serum-free medium in the presence of solvent (white), 10^{-9} M E2 (blue), 10^{-8} M Zea (red) and 10^{-5} M Api (green) alone or in combination with 10^{-9} M E2 (hatched). Cell number was determined by counting. The results are expressed as the percentage of the cell number counted after E2 treatment and are the means \pm SEM of three experiments, Figure S3: Number of ER α /c-SRC interaction /cell after a treatment of 5 minutes with solvent as negative control (white), 10^{-9} M E2 (blue), 10^{-8} M zearalenone (Zea, red) or 10^{-5} M apigenin (Api, green). Interactions were monitored by Proximity Ligation Assay according to manufacturer instruction using anti-ER α (HC-20) (Sc-543) and anti-cSRC (B-12) (Sc-8056) antibodies (Santa Cruz Biotechnology). Results are expressed percentage of controls and are the mean \pm SEM of 3 independent experiments, Figure S4: Analysis of Src (A) and p53 (B) phosphorylation using the Proteome Profiler Kit according to the manufacturer instruction. For Src, cells were treated with solvent as negative control, 10^{-9} M E2, 10^{-8} M Zea or 10^{-5} M Api for 30 min. For the different p53 phosphorylation, cells were treated with 10^{-9} M E2 or 10^{-5} M Api for 24 h. Results are expressed as the relative intensity of specific phosphorylation forms compared to reference spot in arbitrary unit.

Author Contributions: Conception of experiments, S.L., F.D., F.P.; Acquisition of data and analysis, S.L., F.D., T.H.P., S.M.; Bioinformatics analysis, F.C.; Writing—Original draft, S.L., F.C., F.P.; Review, T.H.P., T.E., F.P., Funding acquisition, T.E., F.P.

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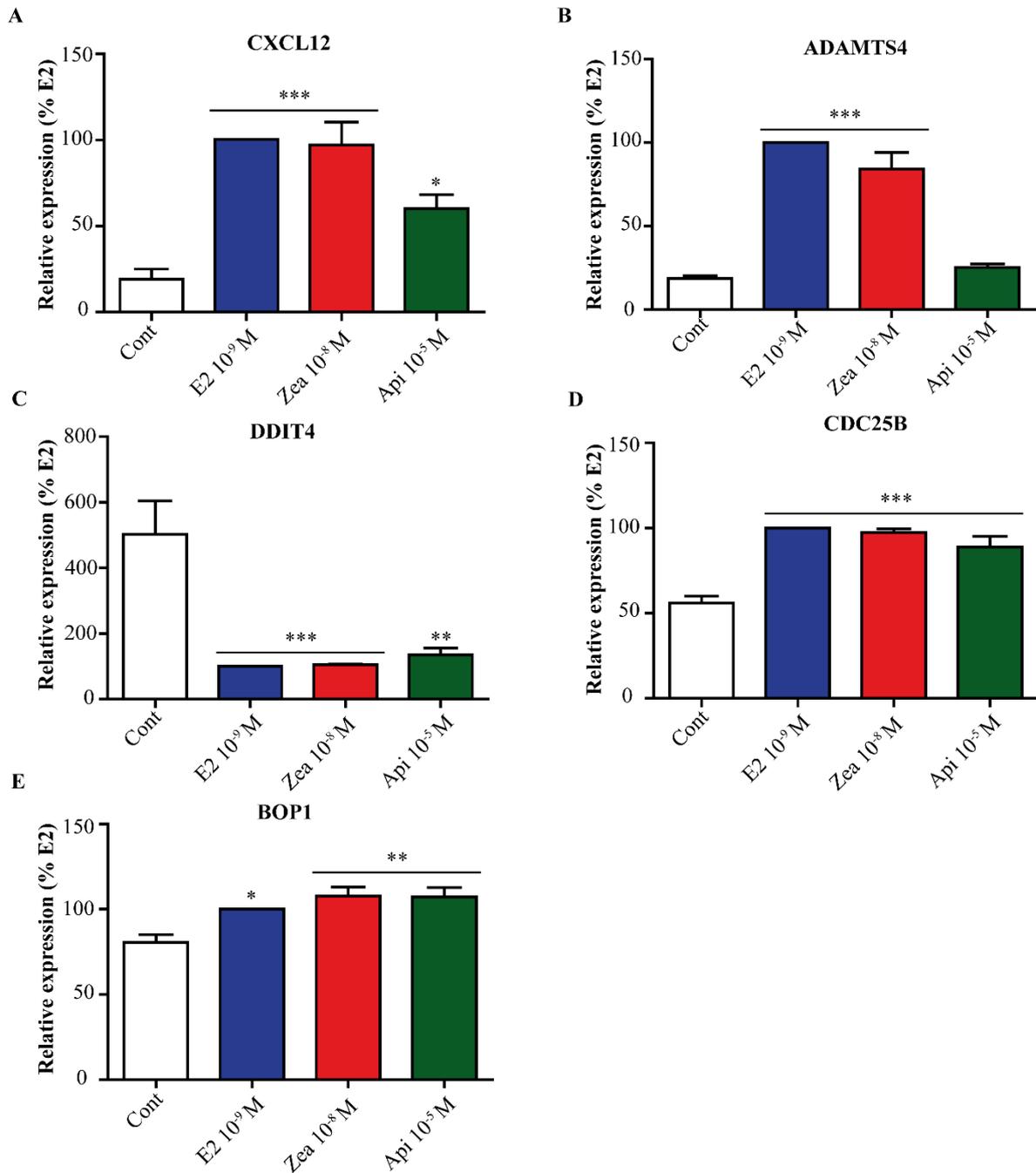


Figure S1. T47D cells were treated with solvent (white) as a negative control, 10⁻⁹ M E2 (blue) as a positive control or with 10⁻⁸ M zearalenone (Zea, red) or with 10⁻⁵ M apigenin (Api, green) for 24 h. The expression of CXCL12 (A), ADAMTS4 (B), DDIT4 (C), CDC25B (D) and BOP1 (E) was assessed by real-time PCR. The expression level of each gene was normalized to the expression levels of the housekeeping genes GAPDH and TBP. The results are expressed as the percentage of the relative expression of transcripts obtained in E2-treated cells and are the means ± SEM of at least three independent experiments.

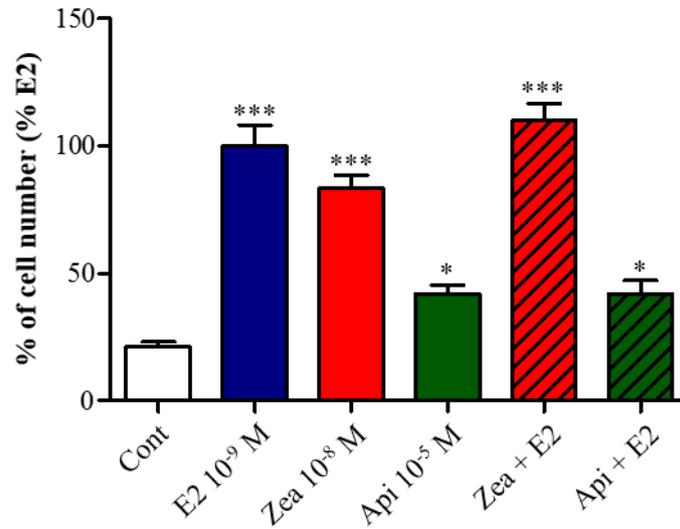


Figure S2. T47D cells were grown for 6 days in serum-free medium in the presence of solvent (white), 10⁻⁹ M E2 (blue), 10⁻⁸ M Zea (red) and 10⁻⁵ M Api (green) alone or in combination with 10⁻⁹ M E2 (hatched). Cell number was determined by counting. The results are expressed as the percentage of the cell number counted after E2 treatment and are the means \pm SEM of three experiments

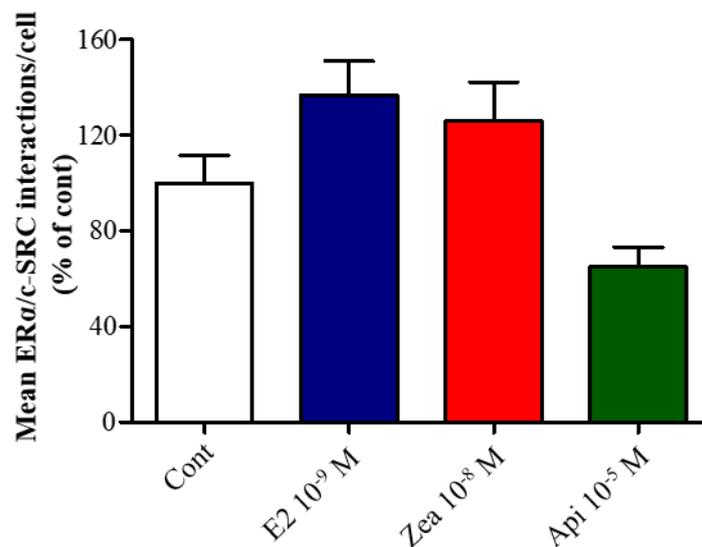
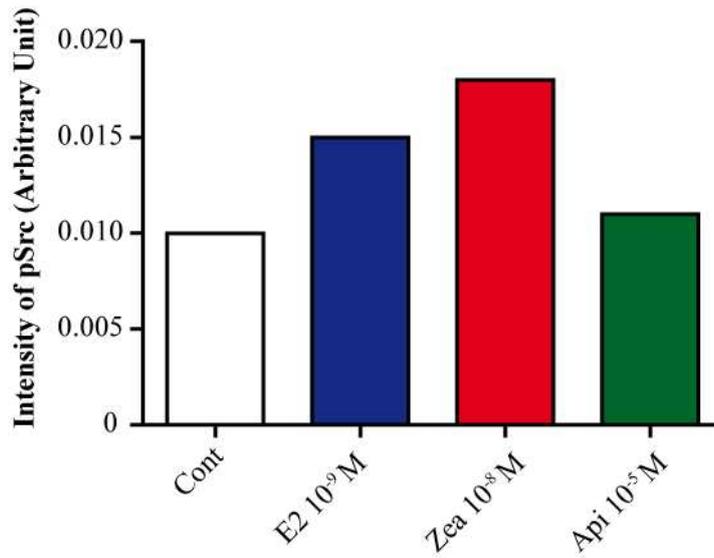


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A



B

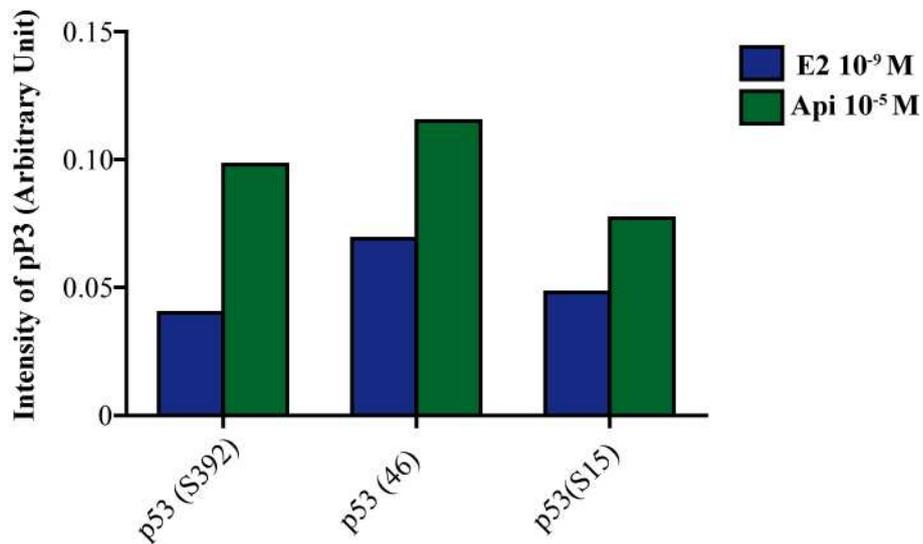


Figure S4: Analysis of Src (A) and p53 (B) phosphorylation using the Proteome Profiler Kit according to the manufacturer instruction. For Src, cells were treated with solvent as negative control, 10⁻⁹ M E2, 10⁻⁸ M Zea or 10⁻⁵ M Api for 30 min. For the different p53 phosphorylation, cells were treated with 10⁻⁹ M E2 or 10⁻⁵ M Api for 24 h. Results are expressed as the relative intensity of specific phosphorylation forms compared to reference spot in arbitrary unit.

**PUBLICATION DE VULGARISATION « What is a
SERM and how does it work? »**

Sylvain Lecomte, **Thu Ha Pham**, Farzad Pakdel

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What is a SERM and how does it work?

Estrogen receptors (ER α and ER β) are nuclear receptors that function as ligand-dependent transcription factors. They play a vital role in many physiological functions such as the development and differentiation of the reproductive tract and mammary gland. They are also involved in bone homeostasis and have a protective role in the cardiovascular and central nervous systems. ERs are also involved in numerous estrogen-associated diseases, including the hormone-dependent cancers (breast, endometrial, ovarian, testis and prostate), as well as metabolic disorders, neurodegenerative diseases, endometriosis, cardiovascular diseases and osteoporosis.

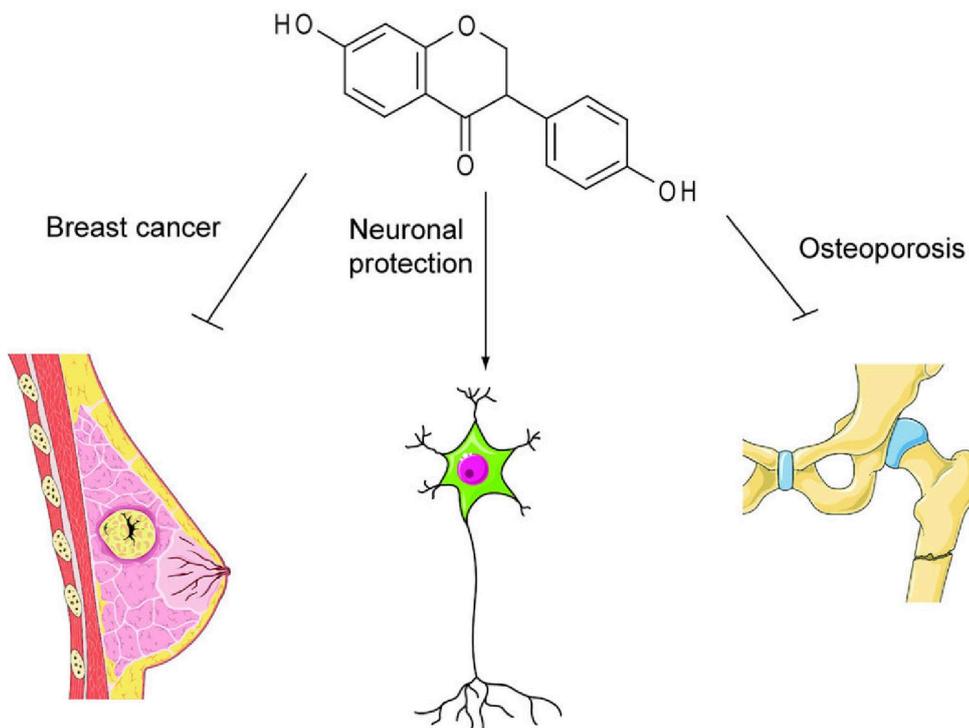


Fig. 1. Characterization of the potential activity of phytoestrogens as SERMs. An ideal phytoestrogen would protect the central nervous system or skeleton and prevent breast cancer without the negative effects associated with hormone therapy.

SERMs (Selective Estrogen Receptor Modulators) are estrogen analogues that bind to ERs but act differently in diverse tissues. In other words, unlike estrogens that act as ER agonists, SERMs have the ability to act selectively as agonists or antagonists depending on a target gene or tissue. For example, they can act as an estrogen in the brain, bone or vessels, but show the opposite action of estrogen in the uterus and in the breast. Hence, these molecules can be used as drugs against osteoporosis, neurodegenerative or vascular diseases, without increasing the risk of cancer of the uterus or breast. Moreover, the anti-estrogenic effect of SERMs that triggers cell growth inhibition is crucial to decrease the risk of ER-positive breast cancer. The specificity of SERMs involves the specific expression of ER subtypes or different co-regulatory proteins or transcription factors in the

various tissues. Differential changes in ER conformational-induced by ligand binding as well as phosphorylation at different ER sites provide also an important mechanism of SERMs to regulate ERs activity. To date, the main clinical applications of SERMs are their use in the prevention and treatment of cancer and osteoporosis. Two SERMs have been evaluated and are currently used in ER-positive breast cancer treatment: tamoxifen (Nolvadex) and raloxifen (Evista). The pure antiestrogen ICI 118,780 (Fulvestrant, sold under the name Faslodex), that shows full antagonistic effect whatever ER-target tissues (Tab. 1), is also used in the treatment of the recurrence or metastatic breast cancers in postmenopausal women.

	Breast	uterus	Bone
E2	+	+	+
Tamoxifen	-	+	+
Raloxifen	-	-	+
ICI 118,780	-	-	-

Tab. 1. Agonist or antagonist activities of ER ligands in E2-target tissues. Compare to 17 β -estradiol (E2), SERMs, such as tamoxifen or raloxifen, are characterized by the duality of their effects on ER-target tissues. They show antagonistic effect (-) in the breast cells, however, an agonistic effect (+) was reported in the bone.

Among the SERM family, phytoestrogens which share common structure with E2, are promising compounds (Fig. 1). Phytoestrogens are second metabolites produced in plants where they exert several functions such as plant defense, flower color, etc. To examine the potential SERM activity of different classes of phytoestrogens, we have performed a screening study in two types of ER-positive cells: the hormone-dependent breast cancer cell lines (MCF-7 and T47D) and a pseudoneuronal PC12 cell line stably transfected with ER α (PC12-ER). Indeed, these models evidence the duality effects of E2 which induces proliferation (MCF-7 and T47D) or differentiation (neurite outgrowth of PC12-ER) depending on the cell context. Therefore, these models provide a complementary tool for characterizing the SERM activity of estrogen analogues. In this work, we showed that despite all the compounds tested induced ER transactivation, they had different impacts on cell phenotype. For instance, resveratrol did not induce breast cancer cells proliferation but potentiated PC12-ER differentiation. Conversely, the mycotoxin zearalenone induced a strong proliferation of MCF-7 and T47D but counteracted the differentiation of PC12-ER. In this work, in addition to the results concerning the SERM activity of phytoestrogens, we described two robust cellular models as complementary tools to characterize the duality effects of estrogenic chemicals.

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Titre : Etude du potentiel des molécules phytochimiques comme adjuvants thérapeutiques dans le cancer du sein

Mots clés : Cancer du sein, AhR, Akt, molécules phytochimiques, glycéollins, apigénine

Résumé : Le cancer du sein (CS) est le cancer le plus fréquent chez les femmes. Pour le CS triple-négatif et le CS résistant à l'hormonothérapie, l'option de traitement est limitée. L'objectif de ces travaux de thèse est d'étudier le potentiel thérapeutique des molécules naturelles, les glycéollins et l'apigénine, contre ces différents sous-types de CS. Les glycéollins sont des phytoalexines produits dans le soja lors de l'exposition à certains éliciteurs. Nous avons montré, pour la première fois, que glycéollin I (GI) et glycéollin II (GII) sont les ligands du récepteur aux hydrocarbures aromatiques (AhR). Ils sont capables de se lier à l'AhR et de présenter des effets agonistes et antagonistes partiels envers ce récepteur. D'autre part, GI et GII inhibent la migration des cellules de CS triple-négatif, MDA-MB-231, en modifiant l'expression des gènes codant pour des facteurs majeurs de la migration et de l'invasion cellulaire telles que la N-cadhérine ou la chimiokine CCL2. L'apigénine (Api) est une flavone trouvée dans plusieurs aliments végétaux. Afin d'étudier l'effet de l'Api dans un contexte de cellules cancéreuses

mammaires hormono-résistantes, nous avons surexprimé, de manière stable, une forme constitutivement active de la protéine Akt dans la lignée MCF-7 (appelé clone MCF-7/Akt). Comme attendu, la croissance du clone MCF-7/Akt est partiellement indépendante de l'œstradiol et, contrairement aux cellules parentales MCF-7, il présente une réponse incomplète à l'anti-œstrogène 4-hydroxytamoxifène, démontrant sa résistance à l'hormonothérapie. Nous avons montré que l'Api a un effet anti-prolifératif sur le clone MCF-7/Akt. De plus, l'Api diminue l'expression de la protéine FOXM1, une protéine impliquée dans le cycle cellulaire, la prolifération cellulaire et la résistance endocrine. L'Api modifie également l'expression des gènes régulés par FOXM1, notamment des gènes du cycle cellulaire, en particulier dans le clone MCF-7/Akt. Ensemble, nos résultats renforcent le potentiel thérapeutique des glycéollins et de l'apigénine. Ces molécules méritent d'être étudiées davantage pour devenir des traitements contre le CS.

Title : Study of the potential of phytochemicals as therapeutic adjuvants in breast cancer

Keywords : Breast cancer, AhR, Akt, phytochemicals, glyceollins, apigenin

Abstract: Breast cancer (BC) is the most common cancer in women. For triple-negative BC and hormone-resistant BC, the treatment option is limited. The objective of this work is to study the therapeutic potential of natural compounds, glyceollins, and apigenin, against these different subtypes of BC. Glyceollins are the phytoalexins produced in soybeans upon exposure to certain elicitors. We have shown, for the first time, that glyceollin I (GI) and glyceollin II (GII) are ligands of the aryl hydrocarbon receptor (AhR). They can bind to AhR and exhibit partial agonist and antagonist effects towards this receptor. On the other hand, GI and GII inhibit the migration of triple-negative BC cells, MDA-MB-231, by modifying the expression of genes encoding major factors of cell migration and invasion such as N-cadherin or the chemokine CCL2. Apigenin (Api) is a flavone found in several plant foods. To study the effect of Api in the context of

hormone-resistant BC cells, we stably overexpressed the form constitutively active of the protein Akt in the MCF-7 cell line (named the MCF-7/Akt clone). As expected, the cell proliferation of the MCF-7/Akt clone is partially estradiol-independent and, unlike the parental MCF-7 cells, it exhibits an incomplete response to the anti-estrogen 4-hydroxytamoxifen, demonstrating its resistance to the hormone therapy. We have shown that Api has an anti-proliferative effect on the MCF-7/Akt clone. In addition, Api decreases the expression of the FOXM1 protein, a protein involved in the cell cycle, cell proliferation, and endocrine resistance. Api also alters the expression of genes regulated by FOXM1, including cell cycle genes, particularly in the MCF-7/Akt clone. Together, our results strengthen the therapeutic potential of glyceollins and apigenin. These molecules deserve to be studied further to become treatments for BC.