



Implications du stress oxydant et du fer dans la cardiotoxicité des anthracyclines et du trastuzumab

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DOCTEUR DE L'UNIVERSITÉ DE BOURGOGNE

Spécialité : Physiopathologie et Pharmacologie Cardiovasculaires

Présentée et soutenue publiquement le 17 novembre 2015

Par Charles GUENANCIA

Né le 14 décembre 1984 à Fontaine-Lès-Dijon

IMPLICATIONS DU STRESS OXYDANT ET DU FER DANS LA CARDIOTOXICITE DES ANTHRACYCLINES ET DU TRASTUZUMAB

Thèse dirigée par le Professeur Luc ROCHETTE

Et co-encadrée par le Professeur Catherine VERGELY

Composition du jury

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Mr le Professeur Atul PATHAK, INSERM 1048, Toulouse

Mr le Professeur Luc ROCHETTE, Université de Bourgogne

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Résumé

Le traitement du cancer a considérablement progressé ces dernières années, permettant une réduction de la mortalité. L'allongement de l'espérance de vie des patients a permis de mettre en évidence l'apparition retardée de toxicités cardiovasculaires induites par ces chimiothérapies. Les mécanismes physiopathologiques à l'origine de ces dysfonctions cardiaques sont complexes, intriqués et restent partiellement méconnus. Une meilleure compréhension des phénomènes impliqués dans ces cardiotoxicités est donc nécessaire afin de prévenir leur apparition. En ce sens, nous avons développé deux approches expérimentales complémentaires visant à comprendre les mécanismes physiopathologiques impliqués dans la toxicité cardiaque des anthracyclines et du trastuzumab.

Une première étude expérimentale a permis de préciser le rôle du fer dans la cardiopathie induite par les anthracyclines. Nous avons démontré qu'une surcharge tissulaire en fer chez la souris, préalable à l'injection de doxorubicine, ne majore pas la cardiotoxicité de cette chimiothérapie. Au contraire, la mise en jeu des défenses anti-radicalaires suite à la charge héminique pourrait limiter les lésions oxydatives cardiaques générées par la doxorubicine. A la vue de ces données, le rôle des chélateurs du fer dans la cardioprotection vis-à-vis des anthracyclines semble remis en question.

Notre deuxième travail expérimental visait à élucider le rôle de la surcharge pondérale dans le développement de la cardiotoxicité des anthracyclines et du trastuzumab. Grâce à un modèle murin de surpoids modéré et de risque cardio-métabolique accru induits par programmation post-natale, nous avons mis en évidence le rôle potentiateur d'une surcharge pondérale sur le développement de la cardiotoxicité aux anthracyclines ; alors que la cardiotoxicité du trastuzumab ne semble pas être en revanche majorée par le surpoids. Nos travaux ont également permis de préciser les conditions dans lesquelles existent des potentialisations des effets lors de l'association doxorubicine et trastuzumab.

Mots clés

Cancer, cardiotoxicité, anthracyclines, trastuzumab, surpoids, stress oxydant.

Abstract

Cancer treatment has advanced considerably in recent years, allowing a reduction in mortality. Longer life expectancy of patients has helped to highlight the delayed onset of cardiovascular toxicity induced by these chemotherapies. The pathophysiological mechanisms responsible for these cardiac dysfunctions are complex, entangled and remain partially unknown. A better understanding of the phenomena involved in these cardiotoxicities is needed to prevent their occurrence. Therefore, we have developed two different experimental approaches to understand the pathophysiological mechanisms involved in the cardiac toxicity of anthracyclines and trastuzumab.

A first experimental study aimed to clarify the role of iron in heart failure induced by anthracyclines. We have demonstrated that a tissular iron overload in mice prior to doxorubicin injection does not increase the cardiotoxicity of chemotherapy. On the contrary, the involvement of anti-radical defenses following the iron load could reduce cardiac oxidative damage generated by doxorubicin. In view of these data, the role of iron chelators in cardioprotection against anthracyclines has to be questioned.

Our second experimental work was to elucidate the role of overweight in the development of anthracycline and trastuzumab cardiotoxicity. Using a mouse model of moderate overweight and of increased risk of cardiometabolic induced postnatal programming, we have highlighted the role of overweight on the development of anthracycline cardiotoxicity; whereas trastuzumab cardiotoxicity did not appear to be increased by overweight. Our work also clarified the conditions in which there are cumulative cardiac alterations when doxorubicin and trastuzumab are associated.

Keywords:

Cancer, cardiotoxicity, anthracycline, trastuzumab, overweight, oxidative stress.

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Synthèse bibliographique

A. Cardiotoxicité des traitements pharmacologiques anti-cancéreux

Dans cette première partie de notre thèse, nous nous attacherons à préciser les caractéristiques et les déterminants de la toxicité cardiaque des médicaments anticancéreux en rappelant certaines données admises et en insistant plus particulièrement sur la cardiotoxicité des anthracyclines.

1. La cardiotoxicité de la chimiothérapie

a) Généralités

La complication cardiaque la plus sévère des thérapies anticancéreuses est l'insuffisance cardiaque congestive. Selon les recommandations nord-américaines, les patients recevant une chimiothérapie cardiotoxique peuvent être considérés comme appartenant au groupe à risque d'insuffisance cardiaque de stade A (1). Un récent consensus d'experts européens et américains a notamment permis de préciser et de classer les dysfonctions cardiaques associées aux thérapies anti-cancéreuses (Cancer Therapeutics-Related Cardiac Dysfunction : CTRCD) (2).

i. Définition de la cardiotoxicité

Est désormais considérée comme CTRCD une chute de la fraction d'éjection ventriculaire gauche (FEVG) de plus de 10 points pour aboutir à une FEVG inférieure au seuil de 53%. Ce chiffre de 53% est considéré comme la valeur seuil de la normalité de la FEVG en échocardiographie bi-dimensionnelle. Les recommandations récentes précisent également que cette chute de FEVG doit être confirmée par d'autres modalités d'imagerie (IRM, scintigraphie). De plus, une nouvelle mesure doit être effectuée deux à trois semaines après le diagnostic de CTRCD, afin de déterminer si la réduction de la FEVG est associée 1) à des signes cliniques d'insuffisance cardiaque (symptomatique) (IC) et 2) son caractère réversible (ou non):

- Réversible : FEVG moins de 5 points en dessous de la FEVG de référence pré-chimiothérapie.

- Partiellement réversible : amélioration de 10 points ou plus de la FEVG depuis le point le plus bas mesuré (nadir) mais restant plus de 5 points en deçà de la FEVG de référence.
- Irréversible : amélioration de moins de 10 points de la FEVG depuis le point le plus bas mesuré (nadir) et restant plus de 5 points en deçà de la FEVG de référence.
- Indéterminée : patient non réévaluable.

ii. *Classification des cardiotoxicités*

Deux types de cardiotoxicité sont différenciés :

- Type I : Lésions doses –dépendantes et irréversibles, avec pour chef de file les **anthracyclines**. L'évolution est classiquement défavorable, mais on peut observer une stabilisation des lésions sous un traitement pharmacologique classique de l'IC (béta-bloquants, inhibiteurs de l'enzyme de conversion de l'angiotensine : IEC). En cas de reprise de la même chimiothérapie, il existe une haute probabilité de récurrence de la dysfonction ventriculaire gauche. D'un point de vue histologique, les lésions observées sont le plus souvent l'apparition de vacuoles cytoplasmiques, une désorganisation myofibrillaire et des lésions de nécrose.
- Type II : Lésions indépendantes de la dose, réversibles, avec comme principal exemple le **trastuzumab**. Il existe de fortes chances de récupération de la fonction cardiaque après interruption de la chimiothérapie, et il est possible de reprendre celle-ci à distance sans à priori déclencher une nouvelle dysfonction cardiaque. La réversibilité totale des fonctions myocardiques reste toutefois sujette à discussions. La validité des résultats cliniques dépendant, comme nous le signalons ultérieurement, des marqueurs diagnostiques retenus. Structuellement, peu d'anomalies histologiques ont été décrites.

Dans ce rappel bibliographique, nous développerons, dans une première partie, les composantes de la cardiotoxicité des anthracyclines puis nous rappellerons les caractéristiques de la toxicité cardiovasculaire :

- Des antimétabolites
- Des agents alkylants
- Des taxanes
- Et de l'hormonothérapie

Dans une deuxième partie de ce document, nous détaillerons de manière plus spécifique la cardiotoxicité des nouveaux médicaments anticancéreux en ciblant plus particulièrement le trastuzumab.

b) **Cardiotoxicité des anthracyclines**

i. ***Incidence et caractéristiques cliniques de la cardiotoxicité des anthracyclines***

L'efficacité des anthracyclines, agents anti-tumoraux intercalants et inhibiteurs des topoisomérases II, les a rendus indispensables dans le traitement de nombreux cancers solides (cancer du sein, ovarien, pulmonaire) mais aussi dans la prise en charge des lymphomes et des leucémies aiguës (3). Cependant, outre leurs effets secondaires communs à toutes les chimiothérapies, les anthracyclines présentent une cardiotoxicité validée par les études cliniques, prise en compte dans l'initiation et la persistance des traitements, et limitant leur utilisation en clinique. La cardiotoxicité des anthracyclines a été initialement décrite avec la Daunorubicine à la fin des années 1960. Elle a par la suite été retrouvée pour toutes les anthracyclines, avec une incidence variable selon la molécule considérée. Trois types de cardiotoxicités peuvent survenir à l'occasion d'un traitement par anthracyclines (4-10):

- une cardiotoxicité aigüe, rare (<1% des patients exposés), qui apparaît pendant ou peu de temps après l'administration du traitement. Elle se traduit cliniquement par des troubles du rythme, aussi bien supra-ventriculaires que ventriculaires, une insuffisance cardiaque, une myo-péricardite ou bien encore des anomalies électrocardiographiques comme des troubles de la repolarisation ou un allongement du

QT. Cette cardiotoxicité est habituellement réversible et non dose-dépendante. Elle ne contre-indique pas la poursuite du traitement par anthracyclines. Des cas de cardiomyopathie aigüe et de mort subite ont toutefois été rapportés.

- une cardiotoxicité subaigüe qui apparaît quelques semaines après le début du traitement et qui se présente cliniquement sous la forme d'une myocardite. Elle est rare mais responsable de 60% de mortalité.

- une cardiotoxicité chronique aux anthracyclines qui se manifeste généralement dans l'année suivant le début du traitement bien que, dans certains cas, son diagnostic ne soit porté que dix à vingt ans après leur administration. On estime que cette cardiotoxicité tardive concerne entre 1,6 et 5% des patients exposés. Elle conduit à une dysfonction ventriculaire gauche souvent sévère, et à l'apparition d'une insuffisance cardiaque congestive. Chez l'adulte, elle prend la forme d'une cardiomyopathie dilatée alors que chez l'enfant, elle conduit plutôt à une cardiomyopathie restrictive. Elle est définie, chez l'adulte, par les critères cliniques et échocardiographiques du consensus d'expert paru en 2014, détaillés dans le chapitre définition ci-dessus (2). Cette cardiotoxicité est dose-dépendante mais il semble aussi exister une susceptibilité individuelle (11, 12). Les principaux facteurs de risque connus de la cardiomyopathie aux anthracyclines sont rassemblés dans le *tableau 1*. Les plus importants sont des antécédents ou des facteurs de risque cardiovasculaires, l'association de cette chimiothérapie à une radiothérapie médiastinale, à d'autres chimiothérapies ou immunothérapies, les âges extrêmes de la vie et le sexe féminin (7, 9, 13, 14). Cependant, l'incidence de cette cardiopathie est extrêmement variable selon les études du fait de la grande variété de marqueurs diagnostiques utilisés.

Tableau 1. Facteurs de risque de la cardiotoxicité aux anthracyclines et molécules apparentées.

Facteur de risque	Augmentation du risque
Age au moment du traitement	Enfants Personnes âgées
Sexe	Féminin
Antécédents cardiovasculaires	Hypertension artérielle Coronaropathie Insuffisance cardiaque Valvulopathie Antécédent de cardiotoxicité
Comorbidités	Diabète Obésité Insuffisance rénale, respiratoire Sepsis
Mode d'administration	Injection intraveineuse rapide
Forme galénique	Moindre risque si liposomale
Dose cumulée	Dose supérieure à : – daunorubicine 550-800 mg/m ² – doxorubicine 400-550 mg/m ² – épirubicine 900-1000 mg/m ² – idarubicine 150-225 mg/m ² – amsacrine 580 mg/m ² – mitoxantrone >100-140 mg/m ²
Irradiation médiastinale	Irradiation médiastinale précoce Irradiation médiastinale cumulée >30 Gy Irradiation concomitante à une dose de doxorubicine cumulée > 450 mg/m ²
Chimiothérapies antimitotiques associées	Association à - cyclophosphamide - taxanes (docetaxel, paclitaxel) - trastuzumab
Perturbations hydroélectrolytiques	Hypocalcémie Hypomagnésémie
Autres	Trisomie 21

ii. *Physiopathologie de la cardiotoxicité des anthracyclines*

Sur le plan physiopathologique, l'étude histologique de biopsies endomyocardiques de tissus atteints met en évidence des altérations caractéristiques : déformation du réticulum sarcoplasmique, formation de vacuoles cytoplasmiques, distorsions myofibrillaires, raréfaction des myofibrilles associées à une nécrose myocardique (15). De nombreuses études se sont intéressées aux incidences à court terme et à long terme des anthracyclines sur la structure et les fonctions des membranes cellulaires, mais aussi des membranes limitant les organites cellulaires. La dynamique des membranes cellulaires est associée à un renouvellement de leurs composants et en particulier des phospholipides. Dans ce cadre, il est important de souligner que les anthracyclines ont une très grande affinité pour certains phospholipides, en particulier la cardiolipine (16). Le rôle de la cardiolipine cellulaire est multiple et encore relativement mal défini. Ce phospholipide se trouve essentiellement dans la membrane interne de la mitochondrie. Les cellules myocardiques étant très riches en mitochondries, on assiste à une accumulation d'anthracyclines du fait de cette affinité. Ce constat, ajouté à de moindres capacités des défenses anti-radicalaires, pourrait expliquer la susceptibilité accrue du cœur à la toxicité des anthracyclines, comparé à d'autres organes. De plus, le métabolisme de la cardiolipine est impliqué dans de nombreuses étapes de la fonction mitochondriale, et notamment dans l'initiation du processus d'apoptose. Lors de la phase initiale de l'apoptose, la cardiolipine est peroxydée, simultanément à la stimulation des récepteurs de mort et à la génération d'espèces réactives de l'oxygène (17). Cette peroxydation interfère avec le fer héminique du cytochrome C et engendre son relargage. En outre, le cytochrome C peut lui-même contribuer à catalyser la peroxydation lipidique de la cardiolipine (18).

La physiopathologie de la cardiotoxicité des anthracyclines fait intervenir le métabolisme du fer et le stress oxydant, dont l'impact sera étudié plus précisément au chapitre B de la présente synthèse bibliographique (10, 19). D'autres mécanismes entrent aussi en jeu, récapitulés sur la *figure 1* (4, 5, 9-11, 14, 20-24). Ainsi, récemment, le rôle des topoisomérases II a été évoqué (25, 26). Il existe deux isoformes de topoisomérases II chez les mammifères: la topoisomérase II α et la topoisomérase II β .

L'activité anti-tumorale des anthracyclines implique la topoisomérase II α et conduit à la formation d'un complexe topoisomérase II α -structure quaternaire de l'ADN. L'isoforme II α est présente uniquement dans les cellules à fort taux de réplication et donc dans les cellules tumorales, contrairement à l'isoforme II β exprimée dans le tissu cardiaque de l'adulte (25). La cardiotoxicité des anthracyclines serait alors partiellement secondaire aux altérations de l'ADN induites par l'interaction entre ces molécules et la topoisomérase II β . Ce mécanisme conduirait à la répression de l'expression de gènes de facteurs de transcription impliqués dans la survie cellulaire et dans la synthèse de protéines du sarcomère (24). De plus, cette interaction anthracyclines-topoisomérase II β pourrait être à l'origine d'une diminution de l'expression génique de la calcium-ATPase du réticulum sarcoplasmique (SERCA) conduisant des perturbations des flux calciques à l'origine d'une diminution de la contractilité myocardique.

Dans le cadre des mécanismes cellulaires mis en jeu dans l'initiation, le développement et la persistance de la cardiotoxicité qui caractérise les anthracyclines, une attention particulière s'est portée sur les toxicités potentielles de certains dérivés du métabolisme des anthracyclines. En ce qui concerne la doxorubicine, son métabolite essentiel, le doxorubicinol (27) qui résulte de la réduction de la chaîne latérale du groupe carbonyle, perturberait les processus énergétiques de la cellule, et aboutirait à l'altération d'intégrité des membranes cellulaires et à une altération des mouvements ioniques, conduisant à une accumulation de calcium intracellulaire responsable d'une perte de l'homéostasie calcique (24). Il en est de même pour l'épirubicine. L'épirubicinol formé à partir de l'épirubicine sous l'action d'enzymes NADPH-oxydases serait capable d'inhiber certaines calcium-ATPases membranaires du réticulum sarcoplasmique conduisant à des anomalies des flux calciques (28). Il a par ailleurs été montré que les anthracyclines perturberaient les flux électrolytiques au niveau des membranes des myocytes : en plus des lésions mitochondriales, il a été décrit une augmentation du courant calcique dans le réticulum sarcoplasmique associée à une inhibition fonctionnelle du réticulum sarcoplasmique et une diminution de l'activité de la pompe Na/K ATP-ase (27). Une sous-expression myocardique du TNF- α (29) et la fixation de la doxorubicine sur des cations bivalents (Ca²⁺, Mg²⁺, Cu²⁺ et Zn²⁺) pourrait également contribuer à ces effets cardiaques délétères (30). Une réponse auto-immune dirigée contre la membrane des cardiomyocytes, dont le fonctionnement serait perturbé par les anthracyclines,

amplifierait aussi la cardiotoxicité de ces molécules (13). D'autres mécanismes semblent participer à cette toxicité cardiaque, comme l'activation de facteurs de transcription conduisant à l'initiation des mécanismes d'apoptose cellulaire.

Figure 1 : Mécanismes physiopathologiques de la toxicité des anthracyclines dans le cardiomyocyte.

Figure tirée de notre revue de la littérature (Rochette *et al.*) (31) dont le texte intégral est disponible dans la suite du manuscrit. Les radicaux libres générés par les anthracyclines sont issus de mécanismes enzymatiques et non-enzymatiques. L'ajout d'un électron à la structure quinone de la doxorubicine induit la formation d'une semi quinone qui se régénère en quinone par la réduction de l'oxygène en espèces réactives de l'oxygène (ROS) telles que l'anion superoxyde. Ce cycle est entretenu par les oxydases et l'endothelial nitric oxide synthase (eNOS). La doxorubicine sous forme de quinone accepte un électron. Cette réaction génère un radical libre semi-quinone DOX•. Le radical superoxyde ainsi formé est converti en peroxyde d'hydrogène (H₂O₂) par la superoxyde dismutase (SOD). La génération non enzymatique de radicaux libres par la doxorubicine résulte de la formation d'un complexe associant la doxorubicine et le fer ferrique (DOX-Fe³⁺) qui subit une réduction, aboutissant à la production du radical superoxyde. Le donneur d'électron pour cette réaction est le glutathion (GSH). Les anthracyclines entrent dans la mitochondrie, entraînant le relargage de l'oxydase du cytochrome C et affectant sélectivement la phosphorylation oxydative. Ces thérapies anticancéreuses engendrent la mort des cardiomyocytes par l'induction de l'apoptose cellulaire, la réduction de la production d'ATP par la mitochondrie, et la génération d'espèces réactives de l'oxygène. Les anthracyclines prolongent aussi la durée d'ouverture des canaux calciques dans le réticulum sarcoplasmique (SR) et agissent sur les canaux de type L.

ATP, adenosine triphosphate; CAT, catalase; DOX, doxorubicin; GPx, glutathione peroxidase; GSH, glutathione; GSSG, oxidized glutathione disulfide; H₂O₂, hydrogen peroxide; MnSOD, manganese superoxide dismutase; NOS, •NO synthase; NOX, NADPH oxidase; O₂⁻, superoxide; ONOO⁻, peroxynitrite; RNS, reactive nitrogen species; ROS, reactive oxygen species; SOD, superoxide dismutase; eNOS, endothelial nitric oxide synthase or NOS-3; •NO, nitric oxide; •OH, hydroxyl radical; RyR, ryanodine receptor; SR, sarcoplasmic reticulum.

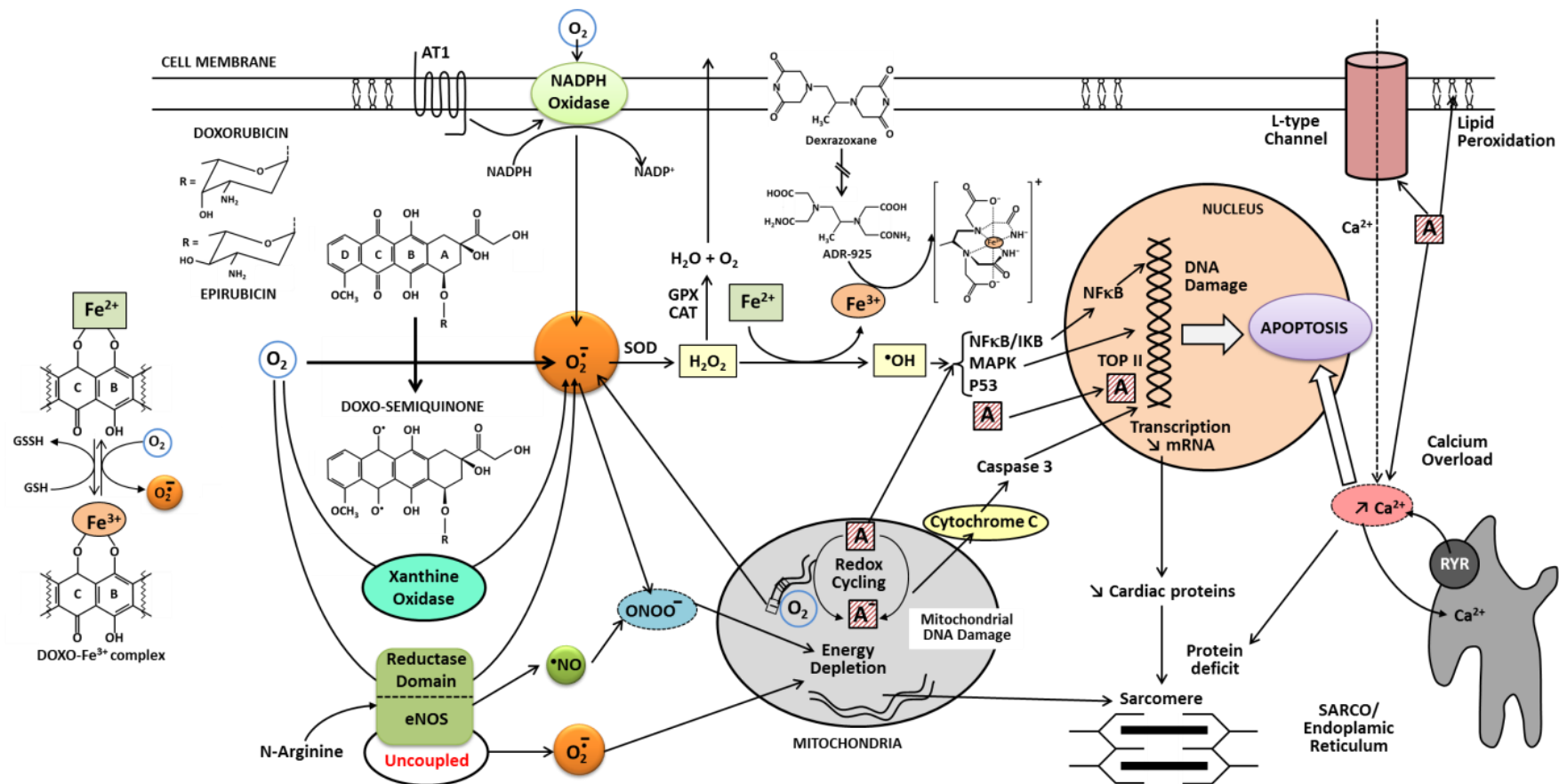


Figure 1. Mécanismes physiopathologiques de la toxicité des anthracyclines dans le cardiomyocyte.

iii. Prévention de la cardiotoxicité des anthracyclines

Initialement, la cardiotoxicité chronique des anthracyclines était considérée comme irréversible et son pronostic apparaissait donc particulièrement sombre. Deux voies de recherche ont été développées afin de lutter contre cette cardiotoxicité : l'une ayant comme objectif de prévenir son apparition et l'autre ayant pour but de traiter cette cardiomyopathie.

Dans le cadre de la prévention et avant toute nouvelle intervention thérapeutique, il apparaît impératif de respecter les contre-indications cardiaques à l'administration d'anthracyclines. Ces contre-indications font partie des recommandations de la Société Française de Cardiologie (32). En cas de FEVG inférieure à 35%, l'administration d'anthracyclines est contre-indiquée. Si la FEVG est comprise entre 35 et 50%, il est recommandé d'évaluer la FEVG après chaque cure et d'arrêter le traitement si elle diminue de plus de 10% ou si elle devient inférieure à 30%. Enfin, si la FEVG est supérieure ou égale à 50%, une seconde évaluation devra être réalisée à la dose de 250-300 mg/m² d'anthracycline et, en fonction des facteurs de risque, une 3^{ème} évaluation à 400-450 mg/m² puis après chaque dose avec arrêt du traitement si la FEVG chute de plus de 10% et devient inférieure à 50%. Par ailleurs, afin de limiter le développement de cette cardiomyopathie, il faut tenter de corriger les facteurs de risque cardiovasculaire du patient.

Différentes voies ont été explorées afin de limiter le développement de cette cardiotoxicité (6, 7, 10). Elles reposent 1) sur la modification des schémas d'administration, 2) la réduction de la dose cumulative administrée, 3) l'utilisation d'analogues des anthracyclines, et 4) l'administration de molécules cardioprotectrices (20).

Chronologie d'administration

Différents travaux ont porté sur la chronologie d'administration des anthracyclines. Initialement, il avait été décrit une diminution significative de l'incidence de la cardiomyopathie aux anthracyclines si leur administration était hebdomadaire et non

toutes les 3 semaines (33). Dans des études plus récentes, aucune différence significative n'a été retrouvée dans l'apparition de cette cardiotoxicité en fonction du schéma d'administration. De même, l'intérêt de l'administration continue d'anthracyclines par rapport à la réalisation de bolus reste controversé. En effet, l'administration continue permet de diminuer les pics sanguins d'anthracyclines mais conduit à une exposition du patient plus longue à ces molécules. Plusieurs études montrent en outre que la cardiotoxicité des anthracyclines ne semble pas prévenue par la réalisation d'une perfusion continue.

Nouvelles anthracyclines et formes galéniques

Afin de limiter la cardiotoxicité de la doxorubicine et de la daunorubicine, de nouvelles molécules de la famille des anthracyclines ont été développées (20, 24). Certaines comme la carminomycine, l'esorubicine, la quelamycine n'ont pas montré de bénéfice comparé à la doxorubicine en termes d'altération de la fonction cardiaque (6). D'autres, comme c'est le cas pour l'épirubicine, ont fait la preuve de leur intérêt et ont été commercialisées. En effet, l'épirubicine possède une activité anti-tumorale identique à celle de la doxorubicine mais présente une toxicité cardiaque moindre. Cette propriété permet l'utilisation de doses plus fortes, et donc l'optimisation du traitement anticancéreux. En effet, la dose cumulative à ne pas dépasser pour l'épirubicine est de l'ordre de 900-1000 mg/m² alors qu'elle n'est que de 400-500 mg/m² pour la doxorubicine. Cependant, ces limites ne sont que théoriques et on peut voir apparaître une cardiotoxicité même à des plus faibles doses. D'autres molécules comme l'idarubicine ont été développées dans la lignée de l'épirubicine.

Plusieurs équipes ont axé leur recherche sur la formulation galénique des anthracyclines. Trois formes liposomales sont actuellement sur le marché: la daunorubicine liposomale, la doxorubicine liposomale et la doxorubicine liposomale pégylée (24). Ces formes liposomales permettent d'augmenter les concentrations plasmatiques d'anthracyclines et d'exercer une administration plus ciblée au niveau de la tumeur (15). En effet, l'administration intraveineuse de l'anthracycline au sein de liposomes empêche leur extravasation au niveau de tissus ayant des jonctions capillaires endothéliales serrées comme le cœur ou le tractus digestif. Ils peuvent sortir de la circulation et pénétrer dans

les tissus si les jonctions sont plus lâches ou s'il existe une interruption au niveau des capillaires comme cela est souvent le cas au sein des formations tumorales. Ainsi, grâce aux liposomes, des organes comme le cœur se trouvent moins exposés aux anthracyclines. Il faut ajouter aussi que la forme liposomale subit un délitement plus long qui conduit à un pic plasmatique moins élevé. La doxorubicine liposomale pégylée (polyethylene glycol-coated) présente alors une demi-vie plus longue par rapport aux deux autres formes, du fait de son revêtement qui diminue sa phagocytose par les monocytes macrophages. Ces trois formes liposomales diminuent le développement d'une cardiomyopathie aux anthracyclines sans pour autant modifier la réponse anti-tumorale, le taux de rémission ou la survie sans récurrence du cancer (34). Parallèlement à l'évaluation clinique, des études histologiques ont été réalisées, et précisent une moindre altération du tissu myocardique lors de l'utilisation de formes liposomales. Le point d'ombre qui persiste est l'imprécision relative quant à la dose cumulative de ces formes à ne pas dépasser au risque de conduire à l'installation d'une cardiomyopathie dilatée toxique.

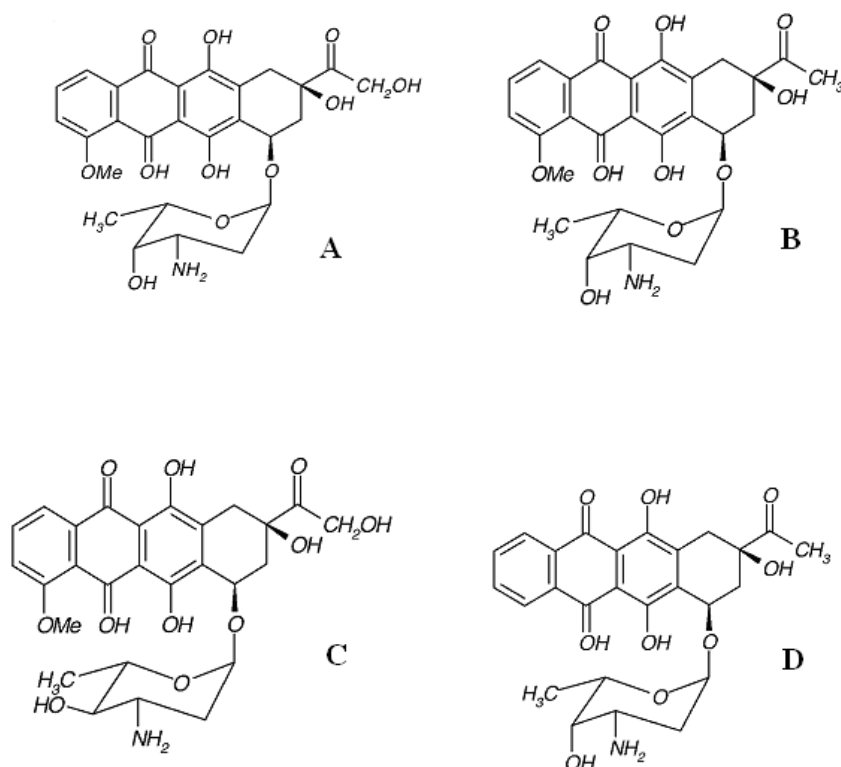


Figure 2. Formules chimiques des anthracyclines.

(A) doxorubicine, (B) daunorubicine, (C) épirubicine, (D) idarubicine d'après Kizek *et al.*(35).

Protection vis-à-vis de la toxicité cardiaque des anthracyclines

Les patients à risque intermédiaire et élevé (selon la présence des facteurs de risque détaillés plus haut) de développer une cardiopathie aux anthracyclines pourraient bénéficier de la mise en route de thérapies préventives cardioprotectrices avant l'initiation ou concomitamment à la chimiothérapie.

1) Dexrazoxane

Comme nous l'avons noté précédemment et comme nous le développerons dans les parties spécifiques de cette thèse, le fer joue un rôle majeur dans les processus impliqués dans la mise en jeu pharmacologique des agents anticancéreux, par ailleurs son rôle est intimement lié aux processus d'oxydoréduction qui règlent de nombreux équilibres métaboliques. De très nombreux travaux ont eu pour but de limiter certaines actions de ce métal fort en préservant les voies associées aux activités antitumorales des anthracyclines. Des chélateurs de métaux, en particulier du fer, ont été synthétisés et testés. Parmi une liste de composés chélateurs de métaux de transition, un dérivé de la dioxopipérazine, aux propriétés voisines de l'EDTA, a été retenu.

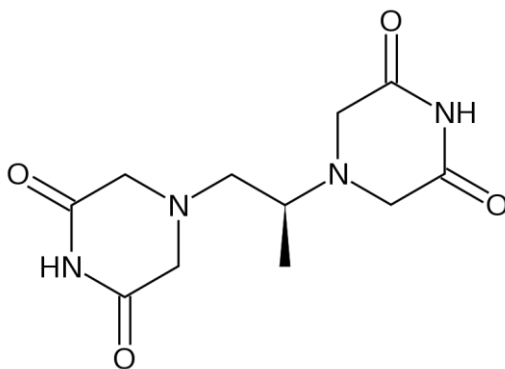


Figure 3. Formule chimique du dexrazoxane.

4-[(2*S*)-2-(3,5-dioxopiperazin-1-yl)propyl]piperazine-2,6-dione

Le dexrazoxane est un chélateur du fer qui prévient la survenue d'une cardiotoxicité dans la majorité des modèles animaux sans compromettre l'efficacité anti-tumorale. Jusqu'à récemment, il était admis que son action protectrice était liée à son effet chélateur, en captant un atome de fer Fe^{3+} de la ferritine, de la transferrine ou d'un complexe fer-anthracycline. Cependant, les mécanismes impliqués dans cette cardioprotection par le dexrazoxane sont autrement plus complexes : bien qu'il s'agisse

d'un chélateur du fer, il semblerait que ses effets protecteurs soient plutôt médiés par une inhibition de l'activité catalytique de la topoisomérase II au niveau cardiaque, ainsi que l'induction d'HIF (hypoxia-inducible transcriptional factor) (36). Cette nouvelle hypothèse expliquerait l'absence d'efficacité clinique d'autres chélateurs du fer (dont l'activité chélatrice du fer est beaucoup plus puissante chélateurs que le celle du dexrazoxane) dans la prévention de la cardiopathie aux anthracyclines, mais aussi la moindre efficacité de l'activité anti-tumorale des anthracyclines en présence de dexrazoxane retrouvée dans certains essais cliniques (37).

Une des études cliniques visant à apprécier l'effet d'un prétraitement par dexrazoxane chez des patientes atteintes de cancer du sein, recevant des cycles de chimiothérapie adjuvante comprenant de la doxorubicine à la dose de 50 mg/m² tous les 21 jours, montrait ainsi que la diminution de la fraction d'éjection (suivie par ventriculographie isotopique) était moins importante, à dose cumulative égale (400-500 mg/m²), dans le groupe recevant le dexrazoxane (2,7 %) que dans le groupe témoin (14,7 %) (38). Une seconde étude, rapportant l'expérience d'un centre recourant systématiquement au dexrazoxane, retrouvait même un taux encore plus bas de cardiotoxicité (1,57 %) (39). Dans un autre essai randomisé en double aveugle de phase 3, 15% des patients du groupe dexrazoxane avaient des effets indésirables cardiaques contre 31% des patients du groupe placebo (p<0,001) (40). Une indication privilégiée du médicament pourrait naître du maintien de son bénéfice lorsqu'il est débuté chez des patients ayant déjà reçu une dose de 300 mg/m² et dont le traitement par anthracyclines reste indispensable. L'utilisation chez l'enfant prévient également l'apparition d'altérations de la contraction ventriculaire gauche appréciée par échocardiographie. L.H. Wexler, en comparant l'évolution de 18 enfants traités par doxorubicine pour sarcome, a montré une meilleure tolérance avec le dexrazoxane (22 % de cas d'atteintes asymptomatiques) que sans (67 %) (41). Ce bénéfice du dexrazoxane permet l'administration de doses cumulées plus élevées de doxorubicine (410 mg/m² versus 310 mg/m²). La méta-analyse de Kalam et Marwick, regroupant 1175 patients issus de 7 essais randomisés, retrouvait un bénéfice au dexrazoxane dans la prévention de la cardiopathie aux anthracyclines important avec une réduction du risque de 0,35 (IC 95% : 0,27-0,45, p <0,00001).

Cependant, malgré ces travaux prometteurs en termes de cardioprotection par le dexrazoxane, il a été ultérieurement suggéré, d'abord chez les enfants puis chez l'adulte,

une augmentation du risque de cancers secondaires, en particulier des cas de leucémie myéloïde aigue et de syndrome myélodysplasique, suite à un traitement par dexrazoxane. Ces résultats n'ont pas été retrouvés dans les essais pédiatriques ultérieurs, mais ont cependant conduit à la restriction de l'indication de ce médicament aux propriétés à visée cardioprotectrice (42).

2) Thérapies de l'insuffisance cardiaque dans le cadre de la prévention de l'apparition de la cardiopathie aux anthracyclines

Lors des dix dernières années, de nombreuses études ont évalué l'intérêt de thérapies classiques de l'insuffisance cardiaque dans le cadre de la prévention de la cardiotoxicité des anthracyclines. La synthèse de ces travaux suggère que ces thérapies seraient partiellement efficaces pour prévenir l'apparition de cette cardiotoxicité. Notamment, la méta-analyse de Kalam et Marwick démontre que les inhibiteurs de l'enzyme de conversion (IEC) et les bêtabloquants diminuent significativement l'incidence des événements cardiovasculaires, sans pour autant préciser quels seraient les patients à qui les administrer prioritairement (37).

iv. Traitement de la cardiopathie aux anthracyclines

Les cardiopathies induites par les anthracyclines ont longtemps été considérées comme réfractaires aux traitements conventionnels de l'insuffisance cardiaque. Cependant, de récents travaux ont démontré l'intérêt d'un traitement par inhibiteurs de l'enzyme de conversion et par bêtabloquants en cas de dysfonction ventriculaire gauche aux anthracyclines (FEVG<45%). En effet, le suivi échocardiographique a démontré une récupération partielle ou totale de la fonction ventriculaire gauche chez plus de la moitié des patients (43). De plus, les recommandations actuelles sur la prise en charge de l'insuffisance cardiaque considèrent comme insuffisance cardiaque stade B la présence de signes cliniques d'insuffisance cardiaque ou une diminution de la FEVG, nécessitant la mise en route des thérapies de type IEC et bêtabloquants (1).

c) **Cardiotoxicités des autres chimiothérapies**

En plus des anthracyclines, de nombreuses chimiothérapies peuvent induire une altération fonctionnelle et structurelle cardiovasculaire. A visée synthétique, et puisque ne constituant pas la thématique principale de cette thèse, nous avons choisi de résumer les propriétés et les conséquences potentielles cardiovasculaires de ces traitements dans le tableau 2 ci-après.

Tableau 2. Propriétés et toxicités cardiovasculaires des chimiothérapies hors anthracyclines.

Classe médicamenteuse	Mécanismes d'action	Composés associés à une toxicité cardiovasculaire	Etudes de la toxicité cardiovasculaire	Mécanismes impliqués dans la toxicité cardiovasculaire	Interactions médicamenteuses
Antimétabolites	Inhibition de la synthèse des acides nucléiques nécessaire à la multiplication cellulaire (44)	-5-Fluoro-Uracile (5-FU) (45)	-Troubles de repolarisation -Ischémie myocardique -Infarctus du myocarde. -Insuffisance cardiaque aiguë -Hypotension (46)	- Spasme coronarien (47) - Phénomènes auto-immuns -Activation de l'agrégation plaquettaire et formation de thrombi - Myocardite - Impuretés fluorées se transformant en composés cardiotoxiques (48)	-Potentialisation de la cardiotoxicité si associée à une chimiothérapie cardiotoxique ou à une radiothérapie concomitante (14, 47, 49)
Agents alkylants	Formation de liaisons covalentes avec les nucléotides de la chaîne ADN entraînant une inhibition de la réplication (50)	-cyclophosphamide -cisplatine - mitomycine C	-Troubles de repolarisation - Troubles du rythme ou de la conduction - Insuffisance cardiaque - Ischémie myocardique (13) - Insuffisance cardiaque - Hypertension artérielle - Accidents vasculaires cérébraux (51) - Insuffisance cardiaque chronique - Microangiopathie thrombotique associant anémie hémolytique, insuffisance rénale et hypertension artérielle	- Phénomènes vasospastiques et thrombotiques au niveau coronaire -Dans les microsomes, cette molécule est réduite en radical semi-quinone qui va subir une oxydation dans les cardiomyocytes. Il en résulte la production de radicaux superoxydes cardiotoxiques (45, 52)	-Potentialisation de la cardiotoxicité si patients préalablement traités par anthracyclines ou ayant bénéficié d'une irradiation médiastinale (45, 52) - Synergie de cardiotoxicité entre les anthracyclines et la mitomycine C (45, 52)

Taxanes	Leur cible moléculaire est la tubuline cytoplasmique dont la polymérisation est nécessaire à la construction du fuseau mitotique. Il en résulte le blocage de la mitose en métaphase (53-55).	-paclitaxel	-Bradycardies -Troubles conductifs sévères - Troubles du rythme	- Effet anti-arythmique de classe I ou IV (52) - Le crémophore EL (agent solubilisant du paclitaxel) peut entraîner une décharge histaminique qui augmente les besoins en oxygène du muscle cardiaque, favorise la vasoconstriction coronaire et aurait des effets chronotropes négatifs (52)	- Le paclitaxel potentialise la cardiotoxicité des anthracyclines (45) par deux mécanismes : -Diminution de l'élimination des anthracyclines -Stimulation la formation de doxorubicinol dans le tissu cardiaque (52, 56, 57).
Hormonothérapie	<p>-Le tamoxifène est un anti-estrogène par inhibition compétitive de la liaison de l'estradiol avec ses récepteurs</p> <p>-Les inhibiteurs de l'aromatase bloquent l'activité de l'aromatase, une enzyme qui convertit les hormones stéroïdiennes en œstrogènes.</p>	<p>- tamoxifène</p> <p>-anti-aromatases</p>	<p>- Thromboses veineuses profondes et embolies pulmonaires - Hypertriglycémie - Accidents vasculaires cérébraux (discuté) (58, 59)</p> <p>- Infarctus du myocarde (60-63).</p>	<p>- Les complications thromboemboliques seraient secondaires à des modifications de la coagulation avec une diminution du taux d'antithrombine III et de protéine S (11)</p> <p>- Augmentation des concentrations sériques de lipides et d'apolipoprotéines majorant le risque de nécrose myocardique (60-63).</p>	

2. La cardiotoxicité des nouveaux traitements anticancéreux

La cardiotoxicité des nouvelles thérapies ciblées implique essentiellement quatre types de molécules. Nous nous attacherons particulièrement à détailler les données cliniques les plus souvent rencontrées, notamment lorsqu'il s'agit d'associations avec les anthracyclines. Sur le plan physiopathologique, nous nous concentrerons principalement sur les mécanismes impliqués dans la cardiotoxicité du trastuzumab, qui constitue l'un des axes majeurs de cette thèse.

a) Cardiotoxicité du trastuzumab

Le trastuzumab (Herceptin®) est un anticorps monoclonal humanisé dirigé contre l'oncorécepteur HER2 (Human Epidermal Growth Factor Receptor-2). Dans 20 à 30% des cancers du sein, on observe une surexpression du récepteur HER2 qui est associée à une durée de survie plus courte (64). Sa mise en évidence utilise des techniques immuno-histochimiques. Le trastuzumab est un anticorps murin humanisé IgG1 dirigé contre le récepteur HER2-NEU du facteur de croissance épidermique humain. C'est un anticorps inhibiteur des tyrosine-kinases. Il ne doit être prescrit que dans les cancers du sein en situation métastatique ou en situation adjuvante et exprimant ce récepteur. Le taux de réponse à cette thérapeutique est de l'ordre de 50%, et il s'agit de la seule chimiothérapie susceptible d'améliorer la survie des patientes souffrant d'un cancer du sein métastatique portant la mutation HER2 (65). Il diminue l'activation des voies de signalisation régulées par HER2 grâce à différents mécanismes :

- en bloquant l'homo ou l'hétéro-dimérisation du récepteur
- par l'augmentation de l'endocytose du récepteur conduisant ainsi à une augmentation de son élimination,
- par son fragment Fc, le trastuzumab possède une activité cytotoxique cellulaire anticorps-dépendante permettant l'inhibition de la prolifération des cellules tumorales humaines qui surexpriment HER2.

Conjointement, en bloquant l'activation du récepteur HER2, le trastuzumab agit sur les voies de signalisation des phosphoinositide 3-kinases (PI3Ks) ce qui conduit à l'inhibition de la prolifération cellulaire et la promotion de l'apoptose. En outre, des études précliniques sur différents modèles animaux ont mis en évidence que le trastuzumab diminuait la vascularisation tumorale en bloquant la néo-angiogenèse.

Lors des études précliniques initiales ou au cours des premières études cliniques chez l'homme, aucun événement cardiaque n'avait été rapporté (4). Or, il est apparu par la suite que cette molécule pouvait induire une cardiopathie toxique (66, 67). Celle-ci est retrouvée chez 1 à 7% des patientes ayant une monothérapie par trastuzumab, chez 2 à 13% des patientes ayant une association paclitaxel-trastuzumab et chez plus de 27% des patientes traitées par une association anthracycline-cyclophosphamide-trastuzumab (63, 67-70). Hormis l'association aux anthracyclines, un âge supérieur à 50 ans, une FEVG infranormale avant traitement et des antécédents cardiovasculaires constituent des facteurs de risque de développement d'une myocardiopathie au trastuzumab.

Au niveau histologique, en microscopie électronique, cette atteinte cardiaque se traduit par des altérations cellulaires aspécifiques telles qu'une hypertrophie des myocytes, l'apparition d'une fibrose interstitielle et une vacuolisation cellulaire focale. Les mécanismes physiopathologiques de la cardiotoxicité du trastuzumab ne sont pas tous élucidés (71). De nombreux travaux sur des modèles *in vitro* et *in vivo* ont permis d'améliorer la compréhension de ceux-ci. L'expression du récepteur HER2 à la surface des cellules est indispensable pour assurer un bon fonctionnement cardiaque (4). Chez le fœtus, ces récepteurs sont présents en grande quantité au niveau du myocarde, et ils permettent le bon développement du muscle et des valves cardiaques (60). Chez l'adulte, l'expression de ce récepteur à la surface des cellules myocardiques est faible, tout comme l'expression de son ARN messager. Il semble que tout stress biochimique exercé sur le myocarde, tel que celui induit par l'administration d'anthracyclines, induise l'expression de HER2 à la surface des myocytes. Ainsi, les altérations chimiques induites au niveau du tissu cardiaque entraîneraient l'expression d'un peptide, la neuréguline qui activerait HER2 en induisant sa phosphorylation, conduisant à l'inhibition de l'apoptose et la promotion de la prolifération cellulaire (60, 67). Le trastuzumab, en se fixant sur ces récepteurs induits au niveau des cardiomyocytes par le traitement chimiothérapeutique, inhiberait cette action et modulerait en plus l'expression

de protéines telles que BCL-XL et BCL-XS (5, 71). Ces protéines jouent un rôle dans les processus impliqués dans l'apoptose cellulaire, BCL-XL comme facteur anti-apoptotique et BCL-XS comme facteur pro-apoptotique (72). Cette fixation du trastuzumab sur HER2 au niveau cardiaque, modifierait aussi les voies de signalisation Akt et NF- κ B qui jouent un rôle important dans la survie des cellules soumises à différentes agressions (66). Il en résulterait une dysfonction mitochondriale, une diminution de la production d'ATP et l'activation du programme apoptotique. Une autre hypothèse concernant la physiopathologie de l'atteinte cardiaque par le trastuzumab reposerait sur une cytotoxicité anticorps dépendante (66). En effet, le lapatinib, petite molécule anti-tyrosine kinase, inhibe l'action du facteur de croissance qui se fixe sur HER2 mais ne possède qu'une faible cardiotoxicité au regard de celle induite par le trastuzumab (4, 71).

A noter que, contrairement à la cardiotoxicité des anthracyclines, la myocardiopathie induite par l'administration de trastuzumab ne semble pas être dépendante de la dose et apparaît partiellement réversible à l'arrêt du traitement, avec ou sans prescription de médicaments de l'insuffisance cardiaque (70-74). Par ailleurs, il semblerait que la reprise du trastuzumab après la récupération d'une fonction contractile normale n'induit pas forcément une nouvelle dégradation de la fonction ventriculaire et l'apparition d'un tableau clinique d'insuffisance cardiaque.

b) Cardiotoxicités des autres nouvelles thérapies anti-cancéreuses

De la même manière qu'au chapitre sur les chimiothérapies, nous avons choisi de synthétiser les propriétés et les toxicités cardiovasculaires des nouvelles thérapies anti-cancéreuses dans le tableau 3 ci-après.

Tableau 3. Propriétés et toxicités cardiovasculaires des nouvelles thérapies anticancéreuses hors trastuzumab.

Classe médicamenteuse	Mécanismes d'action	Composés associés à une toxicité cardiovasculaire	Etudes de la toxicité cardiovasculaire	Mécanismes impliqués dans la toxicité cardiovasculaire	Interactions médicamenteuses
Immunothérapie passive	L'immunothérapie passive non spécifique comprend les différents types d'interférons et l'interleukine-2 (75), qui sont parfois responsables d'atteintes cardiaques.	- interféron α -interleukine 2	- Arythmies supra ventriculaires et ventriculaires - Ischémie myocardique - Spasme coronaire - Infarctus du myocarde - Troubles conductifs - Insuffisance cardiaque - Hypertension artérielle (11, 45, 76) - Augmentation de la fréquence cardiaque et de l'index cardiaque (52) - Troubles du rythme supra-ventriculaires - Ischémie myocardique - Myocardite (45)	- Inconnu, aucun effet cytopathogène direct de l'interféron sur le myocarde n'a encore été mis en évidence - Fuite fuite capillaire majeure avec chute de 40% des résistances vasculaires périphériques, augmentation de la perméabilité capillaire et chute de la pression artérielle (11, 14, 45, 73, 75)	
Anti-tyrosine kinases	Des petites molécules anti-tyrosine kinases agissent en interférant au niveau de la liaison entre les tyrosine-kinases et l'ATP. Elles empêchent le transfert du groupement phosphate de l'ATP au résidu tyrosine. (77)	- lapatinib - imatinib	- Dysfonction systolique - Insuffisance cardiaque (78) - Cardiomyopathie sévère avec altération majeure de la fonction cardiaque (79)	- Le lapatinib inhibe l'activité tyrosine-kinase à la fois des récepteurs ErbB1 et ErbB2. Les mécanismes supposés de sa cardiotoxicité sont similaires à ceux du trastuzumab décrits dans le chapitre précédent - Apoptose et nécrose myocytaire, altération du métabolisme énergétique myocytaire.	- L'incidence des complications cardiaques est augmentée en cas de précédentes administrations d'anthracyclines ou de trastuzumab

		-sunitinib	- Insuffisance cardiaque	- Effet apoptotique sur les mitochondries en activant la caspase-9 (80)	- La co-prescription de molécules cardiotoxiques potentialise le risque d'atteinte cardiaque.
Anti-angiogéniques	Le chef de file des traitements anti-angiogéniques est le bevacizumab qui est un anticorps monoclonal chimère qui se lie au VEGF et inhibe ainsi la liaison à son récepteur (81)	- bevacizumab	<p>- Hypertension artérielle (71)</p> <p>- Insuffisance cardiaque (13, 71, 77)</p> <p>- Evènements thrombo-emboliques (13)</p>	<p>- Diminution de la production de monoxyde d'azote (NO) par la NO synthase endothéliale conduisant à une augmentation des résistances vasculaires</p> <p>- Stimulation de l'expression du Plasminogen Activator Inhibitor-1 (PAI-1) majorant le risque d'hypertension artérielle</p> <p>-Les traitements anti-VEGF diminuent les capacités de régénérescence des cellules endothéliales. Cela favorise l'exposition au flux sanguin du facteur tissulaire et du collagène sous endothélial, qui ne possèdent pas de propriétés anti-agrégantes</p>	

c) **Synthèse des atteintes cardiaques induites par les agents anticancéreux**

Comme nous l'avons vu dans les chapitres précédents, les atteintes cardiovasculaires potentielles liées aux agents anticancéreux sont multiples. Elles sont parfois spécifiques d'une classe médicamenteuse, mais l'on peut également observer une incidence ou des effets différents au sein d'une même classe, selon les molécules administrées. Dans le tableau 4 ci-dessous, nous avons récapitulé, au sein de chaque grande classe médicamenteuse, les effets potentiels sur le système cardiovasculaire, et leur incidence attendue pour chaque molécule potentiellement cardiotoxique.

Tableau 4. Résumé des cardiotoxicités des agents anticancéreux.

Classe de chimiothérapie		Incidence					
Nom de la molécule	Cancer	Arythmies	QT long	Dysfonction systolique	HTA	Ischémie myocardique	Thrombo-embolie
Anthracycline							
Daunorubicine	Leucémie	++/+++	✓	+	-	-	-
Doxorubicine	Sein, lymphome	+/++	✓	++/+++	-	-	✓
Doxorubicine liposomale	Sarcome	+	✓	-	-	+/++/+++	-
Epirubicine	Sein, estomac	-	✓	+/++	-	-	✓
Idarubicine	Leucémie	++/+++	✓	++/+++	-	-	✓
Mitoxantrone	Leucémie	++/+++	✓	++/+++	++	++	-
Agents alkylants							
Cisplatine	Vessie, poumon, ovaire, cerveau	✓	✓	✓	✓	✓	++
Cyclophosphamide	Hémopathies	-	-	✓	-	-	+
Ifosfamide	Col utérin, sarcome	✓	-	+++	-	-	+
Taxanes							
Docetaxel	Sein, poumon	+/++	✓	++	++	++	✓
Nab-paclitaxel	Sein, pancréas	+/++	✓	-	-	-	+
Paclitaxel	Sein, poumon	++	✓	+	-	+	-
Antimétabolites							
Capecitabine	Colorectal, sein	✓	✓	✓	-	++	+/++
5-Fluorouracile	Gastrointestinal	✓	✓	+	-	++/+++	✓
Hormonothérapie							
Abiraterone acetate	Prostate	++	-	++	++/+++	++	-
Anastrozole	Sein	-	-	-	++/+++	++	++
Exemestane	Sein	-	-	-	++	++	+
Letrozole	Sein	-	-	-	++	++/+++	++
Tamoxifène	Sein	-	✓	-	++/+++	++	++
Anticorps monoclonaux							
Bevacizumab	Colorectal	++	✓	+/++	++/+++	+/++	++/+++

Brentuximab	Lymphome	-	-	-	-	+	++
Cetuximab	Colorectal, cerveau	++	-	✓	++	✓	+/++
Ipilimumab	Mélanome	-	-	-	-	-	-
Panitumumab	Colorectal	✓	-	-	++	++	+
Pertuzumab	Sein	-	-	++	-	-	-
Rituximab	Hémopathie	✓	-	-	++	++	++/+++
Trastuzumab	Sein, estomac, ovaire	++	-	++/+++	++	-	+/++
Anti-tyrosine kinases							
Dasatinib	Leucémie	++/+++	+/++	+/++	++	++	+/++
Erlotinib	Poumon	✓	-	-	-	++	++
Gefitinib	Poumon	✓	✓	✓	-	+/++	✓
Imatinib	LMC	-		+/++	-	+++	+
Lapatinib	Sein	✓	+++	++	-	-	-
Nilotinib	Rein	++	++	++	++	✓	++
Pazopanib	LMC	-	-	-	+++	+/++	++
Sorafenib	Rein, foie	+	✓	+	+++	++	+/++
Sunitinib	Gastrointestinal, rein	+	+	++/+++	+++	++	+/++
Vemurafenib	Rein	-	✓	+	++	++	++

+++ : > 10% d'incidence; ++ : 1%-10%; + : < 1% ou rare; ✓ observe mais incidence précise non connue; - : complication non reconnue. HTA : hypertension artérielle ; LMC : leucémie myéloïde chronique.

B. Anthracyclines-trastuzumab : nouveaux aspects des mécanismes moléculaires mis en jeu dans l'initiation et le développement de la cardiotoxicité

Dans la recherche sur le cancer, l'intérêt pour topoisomérase II s'est accru suite à son identification comme cible de thérapies anticancéreuses telles que la doxorubicine. Les anthracyclines sont des agents anticancéreux très puissants, indiqués dans de nombreuses pathologies, et l'identification de cette cible thérapeutique a constitué une étape majeure dans la pharmacologie des médicaments anticancéreux (82). Dans les essais cliniques, la combinaison de plusieurs médicaments différents a souvent été essayée, sur la base de l'hypothèse que ces médicaments agissent sur différentes cibles pharmacologiques avec des actions additives. Une nouvelle génération de médicaments anti-tumoraux a été développée, médiée par des protéines kinases et agissant sur des récepteurs à tyrosine kinase. Diverses thérapies visant ces récepteurs ont été approuvées pour le traitement de plusieurs cancers. Les membres de la famille des récepteurs transmembranaires du facteur de croissance épidermique (famille ErbB) sont des médiateurs puissants de la croissance cellulaire normale et de son développement. La famille ErbB est constituée de quatre types de récepteurs transmembranaires de la tyrosine kinase: le récepteur du facteur de croissance épidermique (EGFR; également nommé HER1), ERBB2 (HER2), ERBB3 (HER3) et ERBB4 (HER4). Les membres de la famille de l'EGFR sont souvent surexprimés dans les tumeurs solides. Les immunothérapies ciblent fréquemment les récepteurs de facteurs de croissance. En bloquant la liaison de ligand et / ou la signalisation par ces récepteurs, des anticorps monoclonaux peuvent servir à normaliser les taux de croissance, induire l'apoptose et / ou aider à sensibiliser les tumeurs aux agents chimiothérapeutiques. Le trastuzumab, le pertuzumab, et l'ado-trastuzumab emtansine sont des anticorps monoclonaux ciblant le domaine extracellulaire et qui sont utilisés pour le traitement du cancer du sein HER2-positif (83). Le rôle majeur de la signalisation ErbB2 dans la physiologie cardiaque est rapidement devenu évident suite au constat que certaines patientes atteintes de cancer du sein traitées par le trastuzumab (Herceptin, anti-ErbB2), un inhibiteur de la signalisation HER2, développaient une dysfonction cardiaque, en particulier lorsque le trastuzumab était combiné avec les anthracyclines. Il est devenu évident que certains de ces agents

anticancéreux ont des cardiotoxicités combinées, et peuvent, au moins chez certains patients, provoquer une insuffisance cardiaque symptomatique et, dans d'autres cas, une dysfonction ventriculaire gauche asymptomatique. Ce risque est d'autant plus significatif lorsqu'il concerne des jeunes patients, guéris d'un cancer traité par anthracyclines, et développant ultérieurement une cardiopathie. Dans la revue de la littérature ci-dessous, publiée en 2015 dans *Trends in Pharmacological Sciences* (31), nous avons abordé les mécanismes de la cardiotoxicité des anthracyclines et du trastuzumab, et détaillé les interactions entre ces deux thérapies aboutissant à la potentialisation des altérations cardiaques. De plus, nous avons précisé le rôle des voies de signalisation médiées par les neurégulines, qui sont des protéines impliquées à la fois dans l'oncogenèse et dans la cardiotoxicité du trastuzumab. En effet, elles régulent les récepteurs ErbB2, site de fixation de cette chimiothérapie, et possèdent des effets anti-apoptotiques et antioxydants au niveau cellulaire.

Anthracyclines/trastuzumab: new aspects of cardiotoxicity and molecular mechanisms

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Anticancer drugs continue to cause significant reductions in left ventricular ejection fraction resulting in congestive heart failure. The best-known cardiotoxic agents are anthracyclines (ANTHs) such as doxorubicin (DOX). For several decades cardiotoxicity was almost exclusively associated with ANTHs, for which cumulative dose-related cardiac damage was the use-limiting step. Human epidermal growth factor (EGF) receptor 2 (HER2; ErbB2) has been identified as an important target for breast cancer. Trastuzumab (TRZ), a humanized anti-HER2 monoclonal antibody, is currently recommended as first-line treatment for patients with metastatic HER2⁺ tumors. The use of TRZ may be limited by the development of drug intolerance, such as cardiac dysfunction. Cardiotoxicity has been attributed to free-iron-based, radical-induced oxidative stress. Many approaches have been promoted to minimize these serious side effects, but they are still clinically problematic. A new approach to personalized medicine for cancer that involves molecular screening for clinically relevant genomic alterations and genotype-targeted treatments is emerging.

Active anticancer drugs: ANTHs and TRZ

In the study of cancer, interest has grown in topoisomerase II (TOP2) following the discovery that it is targeted by active anticancer drugs such as DOX. DOX and derivatives are highly active anticancer agents in many different clinical settings, and the identification of a crucial target of these drugs was a major landmark in the pharmacology of anticancer drugs [1]. One approach that has frequently been used in clinical trials is to combine several different anticancer drugs. The rationale for this combination was originally based on the hypothesis that these drugs act on different pharmacological targets with additive actions. A new generation of antitumor drugs has been shown to be mediated by protein kinases and to act on a family of tyrosine kinase receptors. Various therapies that target

these receptors have been approved for the treatment of several cancers (lung, breast, renal cell carcinoma, and melanoma). Members of the EGF family of transmembrane receptors (ErbB family) are potent mediators of normal cell growth and development. The ErbB family consists of four closely related type 1 transmembrane tyrosine kinase receptors: the EGF receptor (EGFR; also known as HER1), ErbB2 (HER2), ErbB3 (HER3), and ErbB4 (HER4). Members of the EGFR family are frequently overexpressed in solid tumors. Many of the tumor-expressed targets for therapeutic antibodies are growth factor receptors. By blocking ligand binding and/or signaling through these receptors, monoclonal antibodies may serve to normalize growth rates, induce apoptosis, and/or help sensitize tumors to chemotherapeutic agents. TRZ, pertuzumab, and ado-TRZ emtansine are monoclonal antibodies that target the extracellular domain and are used for the treatment of ErbB2-positive breast cancer [2]. The importance of ErbB2 signaling in cardiac physiology soon became evident by the discovery that some breast cancer patients treated with TRZ (Herceptin, anti-ErbB2), an inhibitor of HER2 signaling, develop synergistic cardiac dysfunction, particularly when TRZ is combined with DOX. Some of these anticancer agents have associated cardiotoxicities and can, at least in some patients, cause symptomatic congestive heart failure (CHF) and, in others, asymptomatic left ventricular dysfunction, which is a greater risk in young cancer survivors treated with ANTH. Several mechanisms have been suggested to be associated with the pathogenesis of ANTH-induced cardiotoxicity. Oxidative stress, ion dysregulation, and modifications of the cardiac-specific gene expression cooperate in inducing cardiomyopathy. We review here what is known about the basic mechanisms of cardiotoxicity of cancer therapies with ANTH and TRZ. The identification of targets that mediate cardiotoxicity can also help to guide future drug development.

Oxidative stress and redox signaling

Sources and metabolism of reactive nitrogen and oxygen species (RNOS): reactive oxygen species (ROS) and reactive nitrogen species (RNS)

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Keywords: cardiotoxicity; anthracyclines; trastuzumab; oxidative stress.

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Redox signaling is part of the normal physiology of all cells, and plays a significant role in pathophysiological responses. Cellular (reduction–oxidation) redox environment refers to the reduction potential or reducing capacity in cellular compartments. The redox state refers to the ratio of the interconvertible oxidized and reduced forms of a specific redox couple. Cellular redox status is regulated by the balance between cellular oxidant and reductant levels. Oxidative and reductive stress can trigger redox cascades, and the environment of the cell can determine if a cell will proliferate, differentiate, or die [3]. Any imbalance between oxidants and reductants causes oxidative or reductive stress, which triggers cell damage or aberrant signaling, leading to dysregulation. Oxidative stress occurs during biological processes including cardiovascular disease, atherosclerosis, diabetes, cancer, inflammation, and apoptosis [4].

Free radicals have emerged as important regulators of many physiological and pathological processes through the redox process. High levels of free radical production that overwhelm cellular antioxidant defense systems may damage biomolecules and deregulate signaling pathways.

Free radicals can be defined as molecules or compounds containing one or more unpaired electrons which confer a great degree of reactivity to free radicals. Radicals derived from oxygen (ROS) and nitrogen (RNS: derived from nitric oxide: NO) are the largest class of radical species generated in living systems. RNS are products of cell metabolism and have either beneficial or deleterious effects depending on the concentration reached in the area of the cells [5,6]. RNS include superoxide ($O_2^{\bullet-}$), the hydroxyl radical ($\bullet OH$), carboxyl radical ($CO_2^{\bullet-}$), nitric oxide ($\bullet NO$), and ($\bullet NO_2$), as well as the non-radical species hydrogen peroxide (H_2O_2), hypochlorous acid ($HOCl$), singlet oxygen (1O_2), and carbon monoxide (CO).

The major RNS is endothelium-derived nitric oxide: $\bullet NO$. Under physiological conditions, in the presence of substrate (L-Arginine) and cofactors (tetrahydrobiopterin: BH_4) endothelial nitric oxide synthase (eNOS) produces $\bullet NO$, which is a potent gaseous mediator widely accepted as a key determinant of endothelial function [7]. Of the ROS generated in cells, the major sources of $O_2^{\bullet-}$ include nicotinamide dinucleotide phosphate (NADPH) oxidases, xanthine oxidases and cyclooxygenases (COXs). In addition, the endothelial and neuronal NO synthases (eNOS and nNOS, respectively), both cytochrome p450 reductase-like enzymes, can produce large amounts of ROS when deprived of their key cofactor tetrahydrobiopterin or their substrate L-arginine [7,8]. Of the many ROS-generating enzymes, NADPH oxidase, of which there are 7 homologs (termed Nox 1–5, Duox 1,2), appears to be particularly important in cardiovascular disease [9]. Activation of NADPH oxidases may result from the stimulation of several cell surface receptors, such as the angiotensin II receptor, which is particularly important in hypertension and heart failure. Upon stimulation by angiotensin II, the activity of NADPH oxidases is increased in endothelial and smooth muscle cells, suggesting that in the presence of an activated renin–angiotensin system (either local or circulating), dysfunction due to increased vascular production of superoxide anions is to be expected [10,11].

NO has potent vasodilator, anti-inflammatory, and antithrombotic properties [12,13]. The free radical $\bullet NO$ has a half-life of only a few seconds in an aqueous environment. $\bullet NO$ reacts with molecular oxygen and ROS to generate a range of oxidation products. One well-characterized RNS-forming reaction is that of $\bullet NO$ with superoxide ($O_2^{\bullet-}$), which occurs at nearly diffusion-limited rates to produce peroxynitrite: $ONOO^-$. The latter is itself strongly oxidizing and, when protonated, undergoes homolytic scission to produce $\bullet OH$ and $\bullet NO_2$. Additional reactive radicals derived from other endogenous molecules such as CO and hydroperoxyl can be formed in living systems [14]. Under physiological conditions, scavengers or antioxidants can attenuate the toxic effects of ROS and RNS. An antioxidant can be defined as any substance that, when present at very low concentrations compared to those of an oxidizable substrate, significantly delays or inhibits the oxidation of that substrate. The non-enzymatic antioxidants can be classified further into directly acting antioxidants (e.g., scavengers and chain-breaking antioxidants) and indirectly acting antioxidants (e.g., chelating agents). Antioxidant vitamins (i.e., vitamins C, E, A, and folic acid) are some of the main defense mechanisms of the body's non-enzymatic antioxidant systems. The functions of endogenous antioxidant vitamins are very important via the temporal and spatial monitoring of oxidative metabolic processes [15,16]. Oxidative stress is associated with dysfunction of the mitochondria and endoplasmic reticulum, thus inducing apoptosis and protein misfolding. Mitochondria are endowed with an elaborate and well-defined multilevel antioxidant defense system of enzymes and non-enzymes to quench ROS. The scavenging system includes the matrix Mn superoxide dismutase (SOD), glutathione (GSH), and thioredoxin systems, as well as peroxidases and catalases. Redox homeostasis is crucial for cell viability and normal cell function. Balance is maintained by two major cellular antioxidant systems, namely the glutathione system and the thioredoxin system [17,18].

Several other antioxidants (*N*-acetylcysteine: NAC, lipoic acid) have been shown to reduce lipid peroxidation products [19–21]. Antioxidant defense enzymes such as heme oxygenase 1 (HO-1), catalases, SODs, peroxiredoxins, and GSH peroxidases (GPXs) have been shown to significantly decrease lipid peroxidation products. Glutathione, an endogenous scavenger, exists in both reduced (GSH) and oxidized (GSSG) states. The balance between GSH/GSSG and pyridine nucleotides [NADH/NAD, NAD(P)H/NADP] determines the cellular redox status and the level of oxidative stress [22]. Thioredoxin-dependent peroxiredoxins are the first-line defense against ROS and peroxynitrite ($ONOO^-$) and regulate H_2O_2 -mediated signal transduction. H_2O_2 can act as either a destructive oxidant or as a second messenger [23].

Oxidative stress is able to induce deleterious modifications to a variety of cellular compounds: DNA, lipids, and proteins. ROS can mediate an indirect attack on endogenous molecules by reacting with cellular membrane phospholipids, and this results in the generation of secondary reactive intermediates [24]. Some intermediary metabolites are electrophilic. The nature of electrophilic stress and its role is distinct from and can be functionally

decoupled from oxidative stress. Malondialdehyde (MDA) and 4-hydroxynonenal (HNE) constitute the major products of lipid peroxidation [25]. MDA is suggested to have both mutagenic and carcinogenic effects [26]. HNE is proposed to have an effect on cellular signal transduction pathways. Lipids are susceptible to oxidative degradation caused by radicals, and during autooxidation (peroxidation) the chain reaction is mediated by peroxy radicals and leads to damaged membrane integrity.

Various transcription factors may be activated as a consequence of oxidative stress, leading to the expression of different genes. NF- κ B proteins are a family of transcription factors that play a central role in inflammation, immunity, cell survival, and cell proliferation. In cancer cells, the activity and/or expression of transcriptional factors are deregulated, and these modifications are associated with ROS generation. The Nrf2-Keap1 system is a key factor in cell protection from oxidative and electrophilic insults, and contributes to maintaining the redox cellular microenvironment [27]. Nrf2 binding to antioxidant response element (ARE) not only triggers antioxidant programs that scavenge intracellular ROS but also induces cell proliferation and tumorigenesis [28].

Transition metals and oxidative stress

Transition metals including iron, copper, manganese, zinc, and selenium are at trace levels for normal cellular

function. Iron plays an important role in cell metabolism. Its reactivity allows it to participate in the Fenton reaction as an electron donor to hydrogen peroxide with the subsequent production of \bullet OH. The importance of iron in catalytic processes stems from its redox reactivity which enables it to transition between a reduced ferrous and oxidative ferric state [29] (Figure 1). In the body, an excess or deficiency of metal ions can potentially inhibit protein function and promote oxidative stress [30]. Maintaining cellular iron content requires precise mechanisms for regulating its uptake, storage, and export (Figure 2). The amount of iron absorbed by enterocytes is adapted to body demands. Iron transporters from the SLC11 and SLC40 families are important in intestinal iron absorption as well as whole body iron homeostasis. SLC11(A2) and SLC40(A1) are regulated at transcriptional, post-transcriptional, and post-translational levels. One mechanism involved in iron homeostasis is the post-transcriptional regulator system, which comprises iron responsive elements (IREs) to which iron regulatory proteins (IRPs: IRP1 and 2) bind in an iron-dependent fashion. The IRE-IRP regulatory system is regulated by cellular iron status but also by ROS, eliciting defense mechanisms against iron toxicity and iron-catalyzed oxidative stress [31]. Various cardiovascular disorders and tumorigenesis in multiple human cancer types are related to deregulated iron homeostasis [32–34].

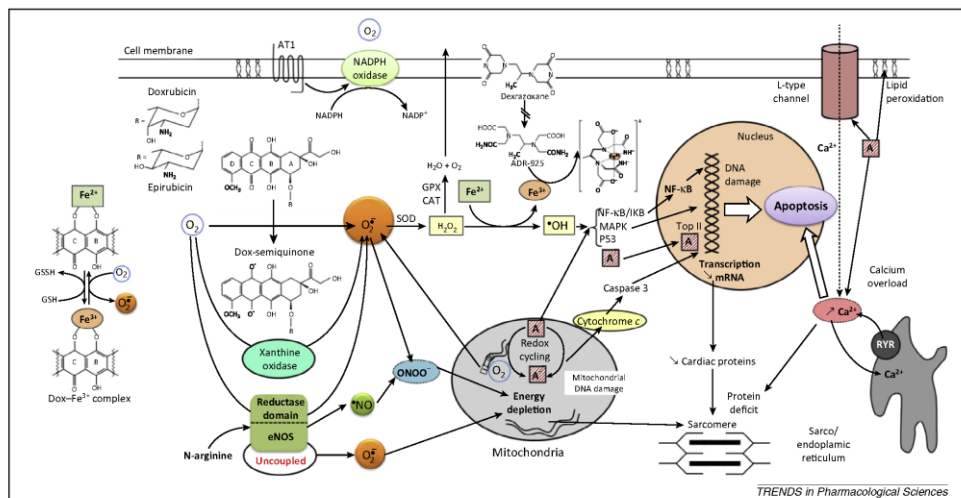


Figure 1. Chemical structures of main anthracyclines (ANTHs): doxorubicin (DOX, also known as adriamycin), epirubicin (EPI), and the 'iron' and free radical hypothesis of ANTH-induced cardiotoxicity. Metabolism: ANTH-generated free radicals by enzymatic and non-enzymatic mechanisms. One-electron addition to the quinone moiety in ring C of DOX induces the formation of a semiquinone that regenerates its origin quinone by reducing oxygen to reactive oxygen species (ROS) such as superoxide anion. This cycle is supported by oxidases and endothelial nitric oxide synthase (eNOS or NOS3) (reductase domain). DOX in the quinone form accepts an electron. This generates a semiquinone free radical: DOX \cdot^- . A superoxide radical is formed and then converted to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD). The non-enzymatic generation of free radicals by DOX results of the formation of a complex associating DOX and ferric iron (DOX- Fe^{3+}) which undergoes redox-cycling to produce superoxide radical. The electron donor for this reaction is glutathione (GSH). ANTH enters the mitochondria, causing the release of cytochrome c oxidase, selectively affecting oxidative phosphorylation. These drugs cause death of cardiomyocytes through induction of cell apoptosis, reduction of ATP production from the mitochondria, and generation of ROS. ANTHs also prolong the opening time of calcium channels in the sarcoplasmic reticulum (SR) and act on L-type channels. Chemistry of dextrazoxane: hydrolysis of dextrazoxane to the intermediate iron-chelating metabolite ADR-925, and then a complex is formed of ADR-925 with Fe^{3+} . Additional abbreviations: CAT, catalase; DOX, doxorubicin; GPX, glutathione peroxidase; GSSG, oxidized glutathione disulfide; MnSOD, manganese superoxide dismutase; \bullet NO, nitric oxide; NOS, \bullet NO synthase; NOX, NADPH oxidase; $O_2^{\bullet-}$, superoxide; \bullet OH, hydroxyl radical; ONOO \cdot , peroxynitrite; RNS, reactive nitrogen species; RyR, ryanodine receptor.

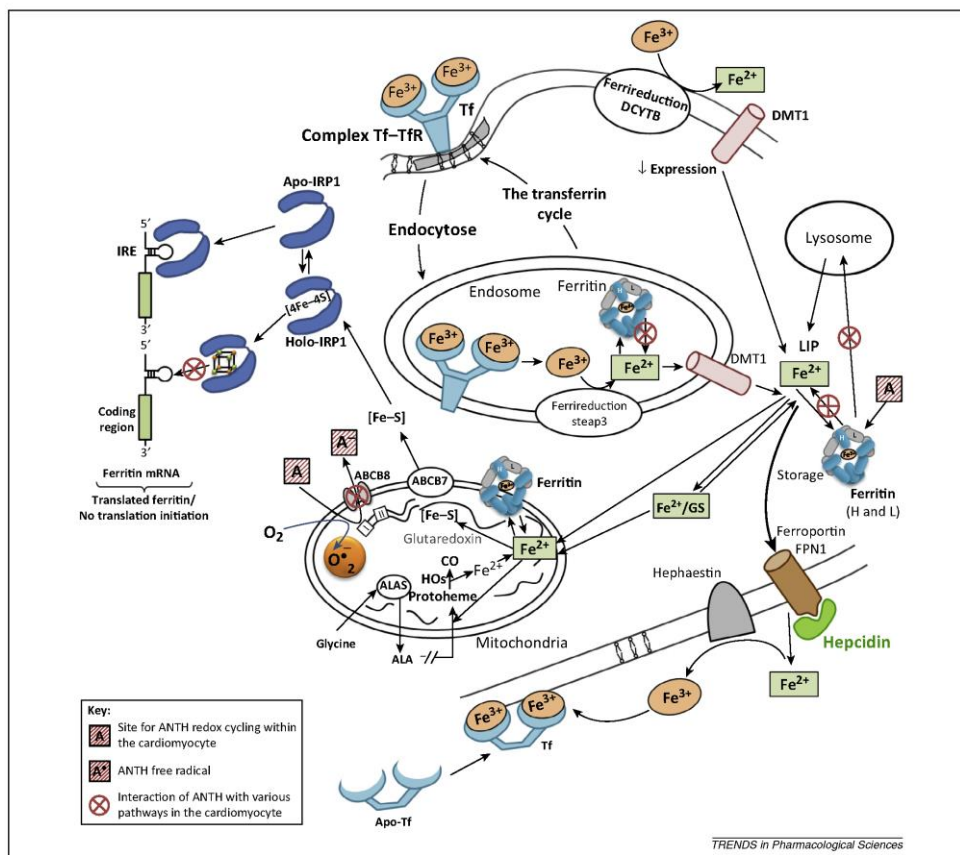


Figure 2. Schema of the effect of anthracyclines (ANTHs) on cellular iron metabolism. Iron is scavenged by transferrin (Tf). TfFe^{3+} binds to the Tf receptor (TfR) on the cell surface and undergoes receptor-mediated endocytosis. The released Fe^{3+} is reduced to Fe^{2+} by the ferrireductase Steap3 within the endosome before export from the endosome by a divalent metal transporter 1 (DMT1). After Fe^{2+} is transported out of the endosome, it is integrated in a specific pool: the labile iron pool (LIP). The major component of the LIP is glutathione-iron conjugate (Fe^{2+}/GS). Fe^{2+} that is not utilized or stored in ferritin is exported by ferroportin (FPN1). Copper-containing ferroxidase hephaestin assists by converting Fe^{2+} to Fe^{3+} , which is then bound by Tf. ANTHs interact with oxidative phosphorylation and iron metabolism in the mitochondria. Inside the mitochondrial matrix, Fe can be directed to different pathways including iron-sulfur (Fe-S) cluster biogenesis. The clusters in the mitochondria are tightly regulated by a transporter: ATP-binding cassette (ABC) transporter (ABCB7). ANTH redox cycling at complex I of the electron transport chain results in the generation of superoxide. ANTH- Fe^{3+} can reduce its chelated Fe through redox reaction, either by oxidation of the side-chain on C9 or the hydroquinone moiety on ring C, forming an ANTH free radical. Heme, the prosthetic group of hemoglobin, myoglobin, and the cytochromes, is generated in the mitochondrial matrix. The first step in mammalian heme biosynthesis is catalyzed by the enzyme 5-aminolevulinic acid synthase (ALAS). The terminal step of heme synthesis is the insertion of Fe^{2+} into the protoporphyrin to produce protoheme. Heme oxygenases (HOs) catalyze the degradation of heme, producing biliverdin, iron, and carbon monoxide. Iron-responsive elements (IREs) are present in the 5'- or 3'-untranslated regions of mRNAs. The iron-regulatory proteins 1 and 2 (IRP-1 and 2) are mRNA-binding molecules. The mRNA-binding activity of IRP1 is regulated by the presence of [4Fe-4S] cluster within the protein. In cells that are iron-depleted, [4Fe-4S] is absent (apo-IRP1) and permits IRP1-IRE binding. In return, if iron levels are high, the cluster forms within the protein (Holo-IRP1) and blocks IRP1-IRE binding. Ferritins and hepcidin are the major regulator proteins. The major target of hepcidin is the protein ferroportin. There is an equilibrium between ferritin-bound iron (Fe^{3+}) and the LIP in cells (Fe^{2+}) by which ferritin prevents the formation of ROS mediated by the Fenton reaction. Key: site for ANTH redox cycling within the cardiomyocyte; ANTH free radical: interaction of ANTH with various pathways in the cardiomyocyte.

RNOS and cellular organelles

In cardiomyocytes, the mitochondria are located near calcium-release sites on the sarcoplasmic reticulum (SR) and can capture a large quantity of the released calcium. Accumulating evidence supports a crucial role for biochemical crosstalk between the SR and mitochondria in normal

cardiomyocyte viability and excitation contraction (EC) coupling. Mitochondrial ROS production can modify excitation-contraction coupling. Several Ca^{2+} channels or transporters, and also myofilaments, are sensitive to redox modifications [35]. Furthermore, mitochondrial ion channels, such as the inner mitochondrial anion channel, the

permeability transition pore, or uncoupling proteins are activated by ROS, thus leading to redox regulation [36].

The endoplasmic reticulum (ER) and mitochondria are the main sources of free radicals (Figure 1). Mitochondria are key regulators of cellular energy and redox metabolism. Several processes, including redox-dependent ATP synthesis by oxidative phosphorylation and ROS production, occur within mitochondria. In mitochondria, when ROS production exceeds local detoxification capacity, oxidative damage to proteins, DNA (mtDNA), and membrane lipids occurs [37]. Each mitochondrion has two specialized membranes dividing the organelle into a narrow intermembrane space (IMS) bordered by the outer mitochondrial membrane (OMM) and the inner mitochondrial membrane (IMM), which encloses the matrix. The OMM is a major source of ROS in particular at complexes I and III, and monoamine oxidases: MAO (mostly the A isoform). Under physiological conditions, the production of ROS is estimated to account for about ~2–5% of the total oxygen uptake by the organism. Oxidative stress accompanied by calcium overload and ATP depletion induces mitochondrial permeability transition (mPT) with the formation of pathological, non-specific mPT pores (mPTP) in the IMM [38]. A specific NOS (mtNOS) was localized in cardiac mitochondria [39]. The ER also plays an essential role in multiple cellular processes including the folding of secretory and membrane proteins, calcium homeostasis, and lipid biosynthesis. The ER supports the biosynthesis of approximately one third of all cellular proteins in eukaryotes. The lumen of the ER provides a special environment to achieve proper folding of proteins [40]. Redox imbalance caused by agents such as chemotherapeutic drugs or pathophysiological conditions leads to the accumulation of unfolded/misfolded proteins in the ER lumen. Various factors, such as oxidative stress and the disturbance of calcium compartmentalization, which interferes with ER function, lead to the accumulation of unfolded proteins. The resulting ER stress triggers the unfolded protein response [41]. The important role of maintaining Ca^{2+} homeostasis within the cell is consequently dependent upon the ER and its many Ca^{2+} -binding chaperones, including heat shock proteins (HSPs) and calreticulin (CRT). CRT, a central Ca^{2+} buffering protein that regulates Ca^{2+} storage and release within the ER, is integral to the quality control of protein folding and Ca^{2+} storage and release within the ER [42] (Figure 1).

Recently, a new concept of immunogenic cell death (ICD) implicating the ER has emerged. The immunogenic characteristics of ICD are mainly mediated by damage-associated molecular patterns (DAMPs), which include factors such as surface-exposed CRT, secreted ATP, and released high mobility group protein B1 (HMGB1). The main function of CRT is in the CRT/calnexin cycle, where it interacts with calnexin and ER protein of 57 kDa (ERp57).

Changes in the redox state and the presence of ROS also affect Ca^{2+} homeostasis by modulating the functionality of ER-based channels and buffering chaperones. In addition, a close relationship exists between oxidative stress and ER stress, both of which may activate signaling events leading to a rebalance of folding capacity and folding

demand, or to cell death [43]. The physical association between the ER and mitochondria, which is known as the mitochondria-associated ER membrane (MAM), plays important roles in various cellular functions. It has clear that the MAM also enables highly efficient transmission of Ca^{2+} from the ER to mitochondria to stimulate oxidative metabolism [44]. An important group of MAM proteins are ER protein-folding chaperones and oxidoreductases; the past decade has seen the association of several MAM proteins with cancer.

RNOS and HSPs

HSPs play an important role in tissue protection. The expression of HSPs is a basic and well-conserved cellular response to an array of stresses. HSPs protect organs against several lesions associated with the increased production of ROS and/or cytokines. In addition, HSPs exert multiple protective effects in inflammation. HSPs are the products of several distinct gene families that are required for cell survival during stress. These include HSP10, HSP20, HSP27, HSP40, HSP60, HSP70, HSP90, and HSP110. The primary factor in HSP transcription is heat shock transcription factor HSF1. The cytoprotective properties of HSP are closely linked to their primary functions as molecular chaperones [45,46]. Overexpression of HSP90 and HSP20 plays an important regulatory role in processes associated with oxidative stress, and leads to a significant decrease in basal levels of ROS [47]. HSP90 plays a two-faced Janus-like role in that it is essential for both normal and cancer cells [48]. HSPs are overexpressed in cancer and contribute to the malignant phenotype and to resistance to therapy [49]. Tumor cells overexpress HSP90 by a factor of two to 10 compared with normal cells of the related tissue. HSP90 is a high-potential targets for cancer therapy and several HSP90 inhibitors are currently in clinical development. One of the most potent oncogenic dependent proteins of HSP90 is ErbB2.

Cardiomyocyte turnover, oxidative stress, and immunity

The proliferation of mammalian cardiomyocytes stops during the first weeks after birth, thus preventing the heart from regenerating after injury. During the ensuing early postnatal period cardiomyocyte replication in the mammalian myocardium becomes undetectable. The number of cardiomyocytes in the mature mammalian heart remains constant throughout life [50]. As a consequence, stress leads to remodeling in which cell death surpasses cell renewal, resulting in progressive heart failure. The association between ANTH exposure in childhood, the development of cardiac dysfunction, and the underlying molecular mechanisms have not been fully elucidated. In the adult heart, mitotic division of cardiac myocytes is undetectable; cardiomyocytes become terminally differentiated. The majority of mature cardiomyocytes are growth-arrested in the G0 or G1 phase. The population of cardiomyocytes was thought to remain stable in number, with a one-to-one ratio to the number of capillary microvessels providing oxygen and substrate delivery, not only during post-natal physiological growth of the heart but also in the adult heart [51]. In these conditions, persistent stress leads to an ultrastructural remodeling in which cardiomyocyte

death exceeds cardiomyocyte renewal, resulting in progressive heart failure. Recently, studies revealed that mammalian cardiomyocytes retain some capacity for division and identified endogenous cardiac progenitor cells in the heart. However, there is no consensus yet about the possibility for new cardiac myocyte generation [52]. The heart is particularly susceptible to oxidative damage because of the specificity of the biology of the cardiomyocyte. Important sources of ROS are present in the myocardium [53] and, paradoxically, antioxidant defenses are less abundant in this tissue than in other tissues such as liver. The activities of three enzymes (SODs, catalases, and GPX) capable of detoxifying activated oxygen were determined in both the heart and liver (Figure 1). Cardiac muscle contains 150-fold less catalase and nearly fourfold less SOD than liver. GPX activities are however similar in the two tissues [54,55]. In the myocardium, as in some other tissues, the reaction of radicals in the presence of O₂, or singlet oxygen, with some amino acids, peptides, and proteins yields hydroperoxides. These species are key intermediates in chain reactions and protein damage. They can be detected in cardiomyocytes and are poorly removed by enzymatic defenses [56].

While the literature clearly indicates the effects of ROS on cardiac contractility, studies on their effects on cardiac excitability are limited. Cardiac excitability depends on the functions of various cardiac sarcolemmal or mitochondrial ion channels, which carry various depolarizing or repolarizing currents that also maintain cellular ionic homeostasis. ROS alter the functions of these ion channels, thus affecting the cellular resting potential and the morphology of the cardiac action potential. Several inward and outward K⁺ channels are affected by different ROS-generating systems. Thus, redox balance regulates cardiac excitability, and under pathological regulation may alter action potential propagation to cause arrhythmia [57]. Exposure of proteins to radicals results in the formation of unstable protein-derived radicals. Protein hydroperoxides play a key role in the propagation of oxidative chain reactions within proteins. These species are capable of inducing strand breaks and mutagenic lesions in DNA, thus inhibiting key cellular enzymes, altering cellular redox status, and depleting antioxidants [58,59]. Protein hydroperoxides are capable of initiating further radical chain reactions both intra- and inter-molecularly, in particular in cardiomyocytes, thus inducing heart failure [60]. The mechanisms of heart failure are complex and multiple, but mitochondrial dysfunction appears to be a crucial factor in the development of this disease. Another mechanism related to oxidative stress and concerning the myocardium is immunity. Although autoimmunity is a well-established pathogenic principle in several endocrine, rheumatic, and neurological disorders, the mechanism has only recently gained more attention in cardiac diseases. Recent studies suggest that the heart possesses an intrinsic system that is intended to delimit tissue injury. It is suggested that this intrinsic stress response is mediated, at least in part, by a family of pattern-recognition receptors (PRRs) that belong to the innate immune system [61]. Depending on individual genetic predisposition, heart-directed autoimmune reactions are thought to

emerge as a consequence of cardiomyocyte injury induced by inflammation, ischemia, or exposure to cardiotoxic substances [62].

The discovery and characterization of the toll-like receptor (TLR) family has led to better understanding of the innate immune system and its function in the different organs and tissues. In addition to being expressed in immune cells, TLRs are expressed in numerous tissues such as those of the cardiovascular system. Many TLRs, including TLR 2, 4, and 9, are expressed in cardiomyocytes. Through these TLRs, cardiomyocytes respond to endogenous or exogenous signals which may influence the pathophysiological responses to dilated cardiomyopathy (DCM) [63]. Moreover, heart failure of diverse etiology is also now recognized as having an important immune component, with TLR signaling influencing the process of cardiac remodeling and prognosis. Hence, the inhibition of TLR signaling may be of great therapeutic benefit in coronary heart failure [64].

HSP20 may play a positive regulatory role in the treatment of DOX-induced cardiomyopathy. DOX treatment was associated with the downregulation of HSP20 in the heart. Overexpression of HSP20 inhibits DOX-triggered cardiac injury, and these beneficial effects appear to be dependent on Akt activation. HSP20 interacts with phospho-Akt, preventing its dephosphorylation, which subsequently maintains BAD phosphorylation and inhibits activation of caspase-3, resulting in the attenuation of DOX-mediated cardiac injury. Thus, targeted therapy to increase HSP20 expression in the heart may hold promise in suppressing DOX-triggered cardiac toxicity [65].

Oxidative stress and cancer cells

The complex series of cellular and molecular changes that contribute to cancer development are mediated by a variety of endogenous and exogenous stimuli, and important among these is the generation of ROS. High levels of ROS generate a chronic oxidative state in the tumor microenvironment which promotes tumor aggressiveness and acquisition of the metastatic phenotype. ROS are generated by 'aberrant activity' of malignant cells [66]. In normal cells, both iron and ROS are carefully managed by the cell to maintain homeostasis or to regulate their functions. However, in cancer cells many of the regulatory processes that control iron and ROS are altered. The especially high iron demand of tumor cells indicates a vulnerable feature of these cells [67]. The persistent oxidative stress of cancer cells is caused by an imbalance between ROS generation and the ability of the cell to scavenge these species. ROS and RNS affect the activity of proteins and genes that respond to stress and which act to regulate genes that are related to cell proliferation, differentiation, and apoptosis. Chronic oxidative stress in tumor cells is influenced by numerous factors such as the deregulation of antioxidant enzymes and mitochondrial dysfunction [66]. Oxidative post-translational modifications have been shown to contribute to cancer. It has been clearly demonstrated that the tumor microenvironment *in vivo* tends to be highly pro-oxidative. The interdependent interaction between tumor and its environment is complex. The effect of oxidative stress at a particular stage of carcinogenesis is directly

proportional to the type and the reactivity of the radicals involved. The initiation of cancer by ROS is supported by the presence of oxidative DNA modifications in cancer tissues [68].

NO is involved in various physiological functions and its role in tumorigenesis has been well studied. There are markedly conflicting findings in the literature regarding NO and its role in carcinogenesis and tumor progression [69]. NO is generated by isoforms of NOS that are widely expressed and sometimes upregulated in human tumors. NO is a cytotoxic or apoptotic molecule when produced at high concentrations by iNOS (NOS-2). The relationship between increased expression of NOS-2 and high angiogenic activity (i.e., microvessel density or VEGF expression) in tumor tissues suggests that cancer-derived NO is associated with tumor angiogenesis [70,71].

The development of the cell death concept is now reconsidered, with special attention to the etiology of apoptosis and necrosis. The immunogenic characteristics of the cell death mode are mediated mainly by molecules such as DAMPs, most of which are recognized by PRRs. Some DAMPs are actively emitted by cells undergoing ICD (e.g., CRT), whereas others are emitted passively (e.g., HMGB1) [72]. NOX activation is initiated by the interaction of immunogens with specific membrane receptors. NOX is identified as a participant in the innate immune response in phagocytic cells, and NO activates specific signal transduction pathways in tumor cells [73,74]. As reported above, the generation of $O_2^{\bullet-}$ and $\bullet NO$ may lead to the production of the harmful molecule $ONOO^-$. It may result in S-nitrosylation and tyrosine nitration of proteins with a concomitant change in their function. $ONOO^-$ is a potent trigger of oxidative protein and DNA damage, including DNA strand breakage and base modification. It activates the nuclear enzyme poly-ADP ribose polymerase (PARP), resulting in energy depletion and apoptosis/necrosis of cells. Peroxynitrite has been associated with the regulation of tumor cell growth, and invasion [75,76].

Inflammation associated with immune reactions or induced by viral infections and/or autoimmune antibodies leads to cardiac remodeling processes with ventricular dilation and systolic dysfunction [77]. Cardiac inflammation is believed to be central during heart failure progression [78]. Fatty acids are ligands of nuclear receptors that affect gene expression, and lipid peroxidation results in the generation of specific structures that are recognized by PRRs of the innate immune system [79]. These include humoral responses, such as naturally occurring autoantibodies (NABs), complement factor H, C reactive protein, as well as cellular receptors such as scavenger receptor CD36 and TLR-4. Thus, oxidation-specific epitopes (OSEs) constitute a novel class of DAMPs targeted by both PRRs and soluble pattern recognition proteins [80]. Impaired NAB function may result in chronic inflammation. In the heart, NAB activation is a major mechanism of ischemia/reperfusion injury [81].

All NOX family members are transmembrane proteins that transport electrons across biological membranes to reduce oxygen to superoxide. In accordance with this preserved function, there are conserved structural properties of NOX enzymes that are common to all family members.

Activation mechanisms and tissue distribution of the different members of the family are markedly different [82]. The physiological functions of NOX family enzymes include cellular signaling, regulation of gene expression, and cell differentiation; NOX enzymes contribute to a wide range of pathological processes such as the regulation of immunomodulation and cellular proliferation [83]. In contrast to the other NOXs, the NADPH oxidase NOX4 exists in the immediate environment of the nucleus. There is accumulating evidence for the involvement of NOX4-derived ROS in genomic instability as well as in cancer and other inflammation-related diseases. Nuclear NOX4-derived ROS may help to regulate interactions between nuclear components and enzymatic activities related to DNA damage signaling and repair [84]. NOX5 mRNA can be detected in all fetal tissues, whereas in adult tissues its expression pattern appears to be more specific. NOX5 was found by immunohistology in endothelial and vascular smooth muscle cells. NOX5 expression is elevated in some cancers and in some cancer cell lines [85].

As reported above, many of the tumor-expressed targets for therapeutic antibodies are growth factor receptors. In this review we summarize the neuregulins/ErbB signaling pathway; this pathway emerging as an important therapeutic target for cancer growth and cardiac-related diseases.

Neuregulins (NRG) and ErbB signaling

The growth factor NRG and molecular mechanisms

In the early 1990s, several groups isolated the proteins encoded by the *NRG* gene, other NRGs, and their receptors [86]. These were named neu differentiation factor (NDF), heregulin, glial growth factor, acetylcholine receptor-inducing activity, sensory and motor neuron-derived factor, and neuregulin. NRGs are a subclass of transmembrane polypeptide growth factors belonging to the EGF family – expressed in the nervous system, the cardiovascular system, mammary glands, the intestine and kidneys. Four distinct genes (*NRG1* to *NRG4*) code for different NRG proteins. The release of NRG-1 takes place at the plasma membrane when the N-terminal ectodomain of pro-NRG-1 undergoes proteolytic cleavage by specific proteases such as β -secretase 1 and ADAM10 (a disintegrin and metalloproteinase domain-containing protein 10). Four NRG genes are found in mammals. NRG isoforms include types I, II, III, IV, V, and VI. *NRG1*, which is located on chromosome 8 in both humans and mice, is the most extensively studied gene [87].

NRGs are widely expressed signaling molecules (Figure 3). NRG-1 is the most extensively studied, particularly at the cardiovascular level. NRG-1 is expressed in the microvascular endothelium [88,89]. NRG transfer their signals through interactions with membrane receptors of the ErbB (EGF receptor) family. In cancer, NRGs act by binding to the human EGF receptor (HER) family of receptor tyrosine kinases: HER/ErbB receptor tyrosine kinases. Other names for NRGs include Neu differentiation factor, or glial growth factors, following some of their biological activities in breast and glial cells. Constitutively active forms of the HER/ErbB receptors have been reported in several tumors and some types of human cancer. Studies in

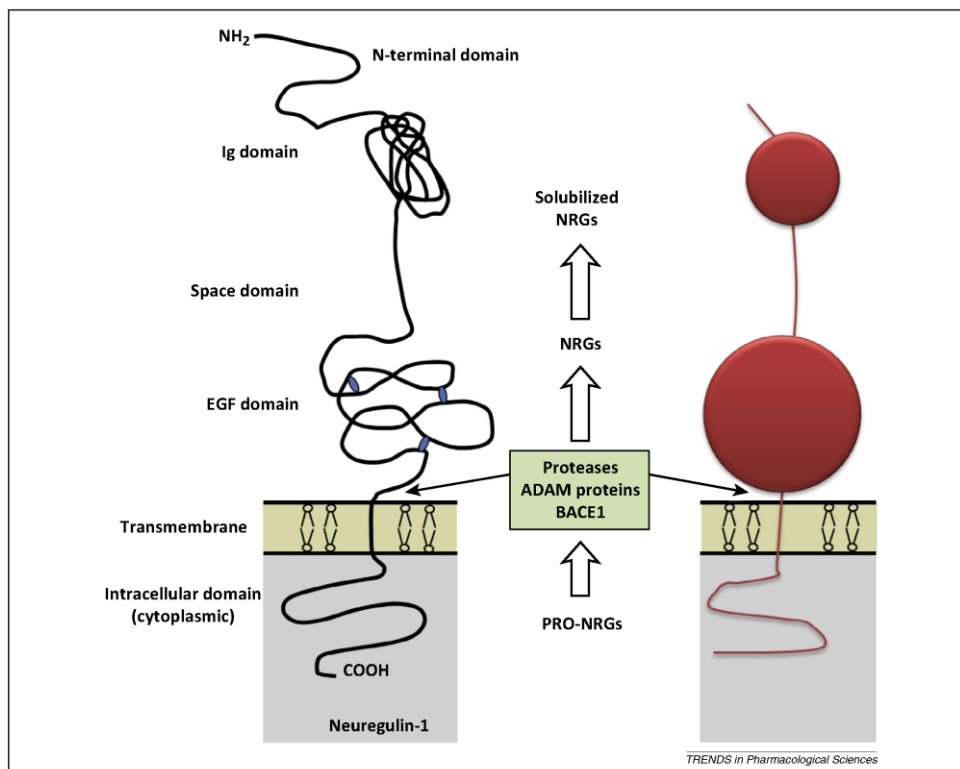


Figure 3. Neuregulin structure. Neuregulins (NRGs) are a subclass of transmembrane polypeptide growth factors of the epidermal growth factor (EGF) family. NRG1 generates six types of protein (I–VI) and at least 31 isoforms. The EGF-like domain is located in the membrane-proximal region of the extracellular domain that is necessary and sufficient for activation of the ErbB receptor tyrosine kinases. NRG1 isoforms are synthesized as transmembrane precursor polypeptides (pro-NRG1s). Once at the plasma membrane, the NRGs may remain at this location as anchored proteins, or may be solubilized. Release of soluble NRGs occurs by the action of membrane metalloproteinase proteases. ADAM (a disintegrin and metalloproteinase) family proteins are membrane-anchored proteases. β -Secretase 1 (BACE1) is an aspartic acid protease.

mice have shown that overexpression of NRGs in the mammary tissue results in the generation of adenocarcinomas [90].

It is now evident that ErbB family members play a prominent role in the initiation and maintenance of several solid tumors. This has led to the development of specific ErbB inhibitors as cancer therapies. The therapeutic approaches for targeting ErbB family members in cancer concern HER2-amplified breast cancer and EGFR-mutant lung cancer. HER receptors include four HER1 receptors (also known as EGFR), HER2 (ErbB2/Neu), HER3 (ErbB3), and HER4 [91]. The structure of ErbB receptors is well known. Figure 4 provides a summary of the NRG/ErbB signaling in the cardiomyocyte and endothelium. HER activation is presented in the Figure 5; for HER2 function, receptor dimerization is required. The extracellular N-terminal domain contains four subdomains (L1:I, CR1:II, L2:III, and CR2:IV) and the leucine-rich subdomains L1

and L2 directly interact with ligand. The cysteine-rich subdomain CR1 contains the dimerization loop responsible for receptor–receptor interaction. A short transmembrane and juxtamembrane domain links the extracellular domain to the bilobed tyrosine kinase domain and the C-terminal tail. Receptor dimerization leads to C-terminal tyrosine phosphorylation. All the ligands of the ErbB receptor family are expressed as single-pass integral membrane proteins. These ligand precursors possess an extracellular component, a transmembrane segment, and a small intracellular portion. The growth factor precursors occur in the extracellular segment and are released by proteolysis mediated by members of the ADAM family. The binding of growth factors to ErbBs promotes dimerization of monomeric receptors and increases the tyrosyl kinase activity of the intracellular domains of ErbBs. The structure of ERBB2 is consistent with its role as a preferred dimerization partner of the other ErbB receptors.

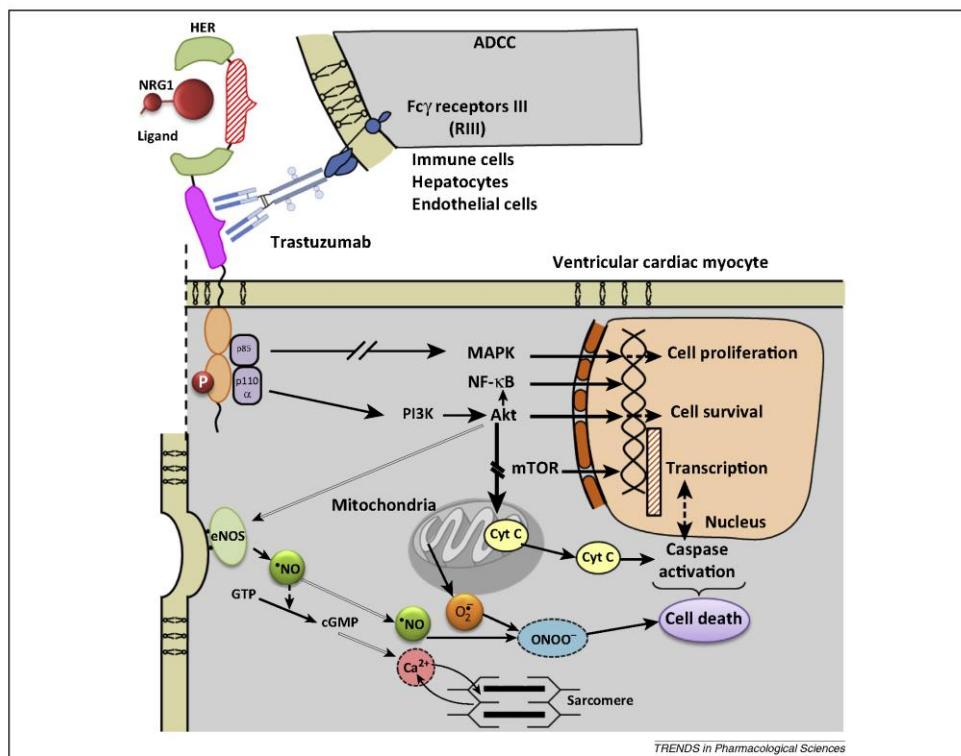


Figure 4. Neuregulin (NRG)/ErbB signaling in the cardiomyocyte and endothelium. NRG-1 is expressed and released by the endocardial and microvascular endothelium. ErbBs are expressed in the ventricular cardiac myocytes. ErbB receptors are also colocalized with eNOS in the caveolae. NRG-1 exerts its effect in a paracrine manner via the ErbB receptors. Heterodimers are stimulators of downstream pathways such as PI3K/Akt and MAPK. Trastuzumab activity is associated with the inhibition of these ways, leading to an increase in cell cycle arrest, suppression of cell proliferation, and cell survival. A mechanism of trastuzumab is to attract immune cells to tumor sites that overexpress HER2 by antibody-dependent cellular cytotoxicity (ADCC). Abbreviation: Cyt C, cytochrome c.

Signaling in cardiomyocytes through the ErbB2–ErbB4 heterodimers is essential for cell proliferation during development and for contractile function in the adult. Various signaling pathways such as phosphatidylinositol-3-kinase (PI3K)–Akt are activated in cardiomyocytes as in breast cancer cells. PI3K activation occurs at the cell membrane and initiates intracellular signaling cascades by generating phospholipids. The p110/p85 dimer receives regulatory stimuli from transmembrane receptors via tyrosine kinases. These tyrosine kinases are able to phosphorylate tyrosine residues within activation motifs, often located within the receptors themselves. Alteration in the p85–p110/PI3K complex is one of the most frequent driver mutations in cancer [92,93].

In growth factor signaling, multiple signaling pathways can be activated simultaneously. The two most frequently identified pathways are the PI3K pathway and the MAPK pathway. The HER2–PI3K pathway is the most frequently mutated or aberrantly amplified oncogenic pathway in

cancer [94]. Inhibitors targeting HER receptors and kinases of the PI3K pathway have been developed [95].

NRGs in the heart and vessels

Multiple isoforms of NRG-1 are expressed in microvascular endothelial cells and cardiomyocytes. There are many effects of NRG-1 β on the biology of heart and vascular cells that could contribute to the beneficial effects of NRG-1 β on cardiac function [96]. These peptides play an essential role in the development of the cardiovascular system, including angiogenesis and compensation of cardiac function. The importance of ErbB2 in normal cardiac development and physiology was demonstrated in mice by cardiac-specific knockout of ErbB2. The mice were initially normal, but developed cardiomyopathy as adults [96].

Cardiac microvascular endothelial cells (ECs) in rat culture express multiple type I NRG-1 polypeptides, including both α and β variants, but only β variants are biologically active on cardiac myocytes [97]. Expression of

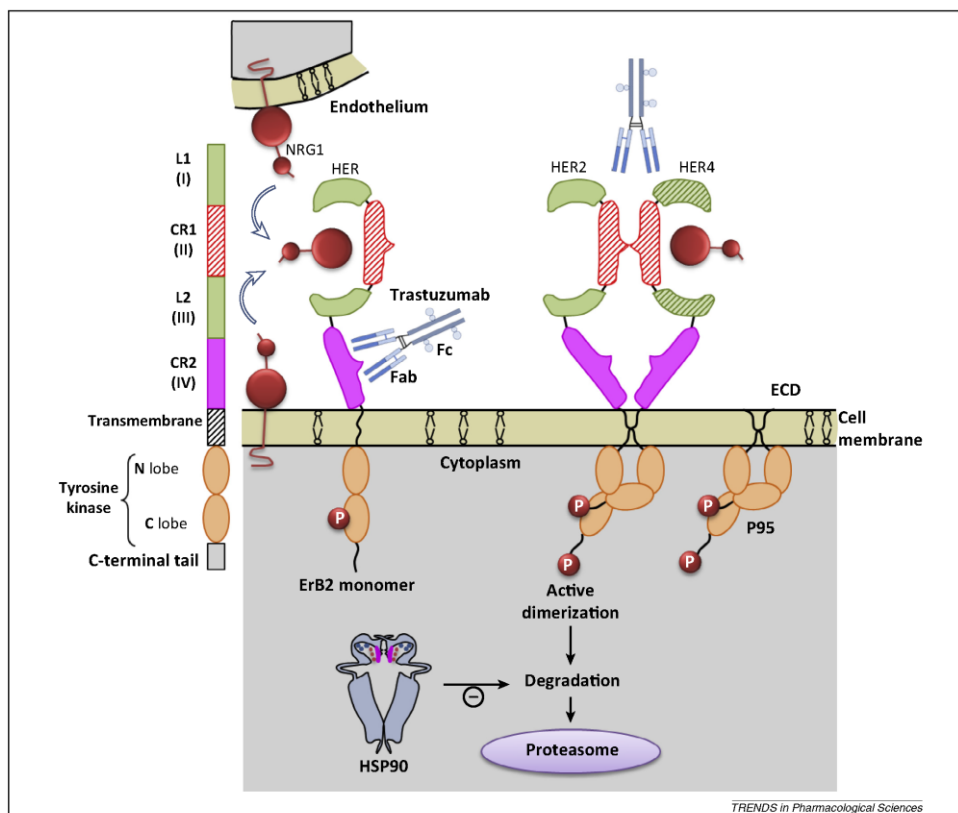


Figure 5. Human epidermal growth factor receptor (HER) activation. HER2 (ErbB2) is a member of the growth factor receptor family that includes four receptors: the epidermal growth factor receptor (EGFR) or ErbB1, HER2 (ErbB2), HER3 (ErbB3), and HER4 (ErbB4). ErbBs, except for ErbB2, consist of an extracellular ligand-binding domain (L1, CR1, L2, CR2), a transmembrane domain, and a cytoplasmic tyrosine kinase domain (N and C lobes). For HER2 function, receptor dimerization is required. With the exception of HER2, ErbB family members are activated by ligand binding to the extracellular domain, which promotes conformational changes that enable the receptors to homodimerize or heterodimerize. Trastuzumab binds to domain IV of the HER2 extracellular domain (ECD) and prevents dimerization between HER2 and HER1, 2, 3, or HER4. HSP90, a chaperone protein, inhibits the proteasome-mediated degradation of HER2 and HER3. Cleavage of the extracellular domain of HER2 generates membrane-bound phosphorylated p95, which is able to activate signal-transduction pathways.

NRGs is upregulated by hypoxia and inflammatory cytokines, such as interleukin 6 (IL-6) and interferon- γ (IFN- γ), in human ECs. NRG- β 1 is thought to play various roles in the prevention of atherosclerosis. NRG- β 1 has antioxidant and antiapoptotic properties, and activates eNOS in cardiomyocytes. Physiological stimuli implicated in the regulation of NRG-1 expression and activity include angiotensin, phenylephrine, and endothelin [98]. An important point is that oxidative stress activates NRG-1 release and activity *in vitro* via a member of the matrix metalloproteinase family. In this field it has been reported that H_2O_2 induced NRG-1 β release from cardiac microvascular endothelial cells in a concentration-dependent manner. NRG-1 β release occurred via proteolytic cleavage of 115 kDa transmembrane NRG-1 β [99] (Figure 3). Upon ligand binding to the extracellular

domain, HER proteins form dimers and mediate potent intracellular signaling. HER2-induced signaling is initiated by an increase in HER2 enzymatic (i.e., kinase) activity and elicits the expression of numerous genes. As a transmembrane protein, the HER2 receptor is a potential target for the physiological process of proteolysis mediated by α -proteases. This proteolysis event leads to the production of two receptor fragments, namely the p105 fragment of the extracellular domain, which is released in the extracellular compartment, and p95HER2 which is embedded in the plasma membrane. The short extracellular domain of the 100–115 kDa p95 HER2 fragment contains five cysteines. At least some of these cysteines establish intermolecular disulphide bonds. The membrane-anchored p95HER2 fragments were active and have been shown to interact with

the full-length HER2 receptor. p95HER2 may be used as a biomarker of an aggressive subtype of HER2-positive breast cancer, and tumors expressing p95 HER2 tend to be resistant to treatment with TRAZ but do respond to lapatinib [100,101].

TRAZ acts by binding to domain IV of the extracellular structure of HER2 causing, among other effects, inhibition of HER2-elicited intracellular signaling and marking HER2-positive cells for antibody-dependent cellular cytotoxicity (ADCC) (Figure 4). The primary cleavage site of HER2 has been located at amino acid position 647–648 and a minor cleavage site has been located at position 644–645. TRAZ targets the ectodomain of HER2. Alternatively, another HER2-targeting drug, lapatinib (a tyrosine kinase inhibitor that competes with ATP for the tyrosine kinase domain of HER2 and induces stabilization and accumulation of inactive Her2), could be used [102].

Two key signaling pathways activated by the ErbB family dimers are the MAPK pathway, which stimulates proliferation, and the PI3K–Akt pathway which promotes tumor cell survival. Activation of the Akt family allows the activation through phosphorylation of many proteins, and this initiates processes to enable tumor cell survival and the suppression of apoptosis and cell cycle control. PI3K inhibitors, such as GDC-0941, could be used in combination with TRAZ-based therapies [103].

Blockade of PI3K–AKT inhibits the phosphorylation of Forkhead box class O (FOXO) family member proteins. FOXO proteins are possible therapeutic targets and putative biomarkers for specific cancers. FOXO factors are translocated to the nucleus, where they repress the transcription of survivin and IL-8, which are members of the inhibitor of apoptosis (IAP) family of proteins [104,105].

NRG-1 β rapidly enhances NO production in adult ventricular myocytes through the activation of the PI3K–Akt pathway and thus protects them from cell death induced by oxidative stress. Akt is able to initiate a change in mitochondrial respiration, thereby decreasing the production of ROS and increasing cell survival. If Her2 signaling is blocked, cardiomyocytes are unable to activate cell survival pathways associated with excess ROS. Therefore, blockage of HER2 enables the accumulation of ROS within cardiomyocytes, which leads to the development of cardiac dysfunction by triggering cardiomyocyte apoptosis.

In summary, several lines of evidence suggest that ErbB2 could be a potential target for anticancer therapy through two mechanisms: first, inhibition of (i) direct antibody binding, (ii) dimerization, (iii) tyrosine kinase activity, and (iv) HSP90; the second mechanism concerns targeting for intracellular drug delivery or through the recruitment of cytotoxic effector cells [106].

ANTHs and cardiotoxicity: chemical properties and mechanisms of action

The potential mechanisms of DOX-mediated cell death
Several mechanisms have been proposed to explain DOX-mediated cell death at clinically relevant doses (~40–60 mg/m²). Several studies have reported that oxidative stress was a major mediator of DOX-induced cardiac gene dysregulation. However, it is also possible that DOX inhibits cardiac gene expression by inhibiting

DNA replication/transcription or by aggravating protein degradation. Many anticancer and antibacterial drugs such as the DOX target TOP (type I and type II) to kill cells. Poisoning of DNA TOPI is the mechanism by which DOX interferes with tumor growth. DOX (also known as adriamycin) belongs to a class of compounds with similar structures, the ANTH group. Like daunorubicin, the first ANTH compound to be described, DOX was isolated from *Streptomyces peucetius*, a soil bacterium [107].

DOX has a high affinity for cell nuclei: as much as 60% of the total intracellular amount of DOX is found in the nucleus. The binding of ANTH to DNA inhibits DNA polymerase and nucleic acid synthesis. In addition, ANTHs stabilize the otherwise cleavable complex between DNA and homodimeric TOP subunits, resulting in the formation of protein-linked DNA double-strand breaks. DOX and other ANTHs [epirubicin (EP), daunorubicin] possess a wide range of clinical indications, including the treatment of solid tumors of the ovary, breast, and gastrointestinal system, and leukemia. DOX is composed of a planar aromatic ring structure containing an anthraquinone chromophore, and a sugar group (daunosamine) (Figure 1). Once taken up by the tumor cell, the planar ring structure is intercalated between adjacent DNA base pairs. A correlation between the ability of DOX to form a covalent adduct with DNA and its cytotoxicity has been found [108].

TOP belong to a family of highly conserved enzymes which are ubiquitously found in all cells. They are essentially involved in the control of DNA topology. The human genome possesses seven TOP genes and include nuclear TOP (TOP1), mitochondrial TOP1 (TOP1 mt), TOP 2 (α and β), and TOP 3 (α and β). Type I can be further subdivided into types IA and IB. Elevated TOP2 expression is present in tumor tissue, this property has stimulated the development of antitumor agents that induce cytotoxicity through TOP2 inhibition and DNA damage [109]. The anticancer activity of DOX is attributable to the killing of dividing cells, where TOP2 α is the major form of the enzyme. An ANTH such as DOX binds to both DNA and TOP2 to form a ternary TOP2–DOX–DNA cleavage complex which triggers cell death. TOP2 β is also a target for DOX, and the TOP2 β –DOX–DNA ternary cleavage complex can induce DNA double-strand breaks (DSBs), leading to cell death. Heart muscle failure is a side effect that results from damage to non-dividing cells, where TOP2 β is the major form. In the presence of TOP2 β , DOX activates the DNA response and apoptosis pathways, and triggers a marked alteration in the transcriptome that selectively affects oxidative phosphorylation and mitochondrial biogenesis in cardiomyocytes.

Cell cycle arrest or apoptosis in response to DNA damage is mediated primarily by the p53 transcription factor. At higher concentrations of DOX, p53 is activated and induces apoptosis through transcriptional upregulation of Bax. Mutations disturbing p53 function have been associated with resistance to ANTH-containing chemotherapy [110].

Mechanisms of cardiotoxicity by DOX: the free radical hypothesis

In addition to causing direct DNA damage by free radical formation, the administration of low doses of DOX can

result in increased levels of oxidative metabolism. Increased oxidative stress due to DOX metabolism has also been regarded as the classical mechanism of cardiotoxicity. The quinone structure of DOX can be oxidized to a semiquinone radical through the addition of one electron, which is mediated by several NAD(P)H-oxidoreductases. Semiquinone radicals quickly react with oxygen to generate superoxide and hydrogen peroxide causing DNA damage. Cardiotoxicity has been attributed to iron-based free radical-induced oxidative stress. DOX has a strong affinity for iron and the iron complex may cause lipid peroxidation through its interactions with the negatively charged membranes. DOX reduction in the presence of free iron also sets up a cycle for free radical generation (redox recycling) and the metabolite doxorubicinol (DOXol) is known to interact with thiol groups on proteins, thus compounding the damage to the cell. Electron paramagnetic resonance spin-trapping studies have shown that among a set of ANTH-Fe(III) complexes only systems in which the drug contains an α -ketol group, DOX-Fe(III) and EPI-Fe(III), were able to reduce Fe(III) and generate hydroxyl radicals under aerobic conditions and in the absence of added reductants. The α -ketol group reduces Fe(III) and induces an increase in hydroxyl radical production. Fe(III) oxidizes the α -ketol group leading to a semidione free radical intermediate, which after a second oxidation step becomes an α -ketoaldehyde. The iron(III) complexes of DOX and EPI were observed to undergo a self-reduction (autoxidation) reaction in the absence of added reductants under aerobic conditions that resulted in the formation of ferrous ANTH complexes [111]. These reactions lead to oxidative stress that will cause cytotoxicity through multiple mechanisms. Mitochondrial dysfunction has been shown after DOX treatment *in vitro*, with cytochrome *c* release leading to caspase-9-mediated caspase 3 activation and resulting in apoptosis.

The relationship between iron and ANTH cardiotoxicity may also be related to disruption of cardiac iron homeostasis which occurs via the targeted interaction of DOX with iron regulatory proteins such as IRP (Figure 2). Pathways linking interactions of IRPs with DOX, DOXol, and quinone-derived ROS have been characterized and have been shown to play a role in cardiotoxicity. ANTHs can interact with iron-responsive element (IRE) regions of mRNAs, thus affecting IRP-mediated regulation of several iron metabolism proteins [112]. It has been shown that ANTHs are able to interact with cellular iron in a more complex way than merely producing ROS. ANTHs decreased the binding of IRPs to the IREs of mRNA, thus modifying the expression of proteins that are crucial for maintaining optimal intracellular iron levels, and iron chelators might also interfere with ANTHs and cellular iron in a more complex way than the Fenton reaction [112,113].

We demonstrated that DOX induced cardiotoxicity through redox cycling, ROS generation, and lipid peroxidation [114]. It has also become apparent that DOX can induce apoptosis via mechanisms that do not directly involve ROS production and oxidative stress, although this point is complicated by the fact that apoptosis itself also generates ROS and RNS. Multiple mechanisms are evidently involved in DOX-induced cardiotoxicity. It is

suggested that calcium dysregulation plays a major role in the pathogenesis of this cardiomyopathy. This cardiotoxicity is accompanied by an increase in intracellular calcium levels. Dysregulation of intracellular calcium concentrations is both a result and a cause of ROS generation. DOX-mediated ROS generation and apoptosis can be inhibited by using a Ca^{2+} antagonist [115]. Mechanisms that have been suggested include alterations in genes important for the structural integrity and enzymatic function of cardiac and vessel myocytes. These phenomena can lead to inadequate maintenance of contractile function in the heart, and we demonstrated that cardiac dysfunction has been associated with the upregulation of fetal genes. DOX-induced cardiotoxicity concerned mRNA modifications in the genes encoding ANF, β -MHC, and SERCA2a in hearts collected 2 months after the end of the DOX treatment. We observed a very significant increase in ANF and β -MHC expression associated with a significant decrease in SERCA2a. A significant positive correlation was found between SERCA2a expression and $+dP/dt$, whereas there was a significant negative correlation between ANF expression and $+dP/dt$ in the hearts [116]. Concerning the role of mitochondria in DOX cardiotoxicity, data indicate that the major mechanism of the disease of the heart is via inhibition of the electron transport chain (Figure 1). There is a rapid response at the transcriptional and translational level of many of the genes coding for proteins of the electron transport chain complexes. Biochemical analysis showed that activity of complexes I to III was reduced while that of complexes IV and V was increased [117].

Other cytotoxic mechanisms have been explored in particular via pharmacological approaches using drugs with antioxidant properties [118]. Despite abundant studies concerning the role of oxidative stress mediated by DOX, it must be noted that the administration of an antioxidant such as vitamin E failed to protect the heart tissue against DOX-induced cardiotoxicity. The preventive administration of sildenafil, a phosphodiesterase 5 inhibitor, can attenuate cardiomyocyte apoptosis and left ventricular dysfunction in a mouse model of DOX-induced chronic cardiotoxicity [119]. In a rat model of DOX cardiotoxicity, the administration of cannabinoid-1 receptor antagonists improved the degree of cardiac dysfunction [120]. Resveratrol pretreatment in acute DOX treatment significantly decreased ROS generation and improved antioxidant enzyme activity. These effects were associated with cardiac protection [121]. The antioxidant efficacy of numerous compounds, such as ebselen (a glutathione peroxidase mimetic), probucol (a vitamin E mimetic), and ranolazine in the prevention of ANTH-induced cardiotoxicity has been demonstrated in various studies [122–124]. ANTH-induced cardiotoxicity is certainly a multifactorial process [125,126]. Pathways of DOX metabolism are also highly relevant in cardiotoxicity. DOXol is more polar than DOX and accumulates at higher levels and for longer times in the heart, and thus exacerbates cytotoxicity [127].

ANTHs, mitochondrial dynamics, and the immune system

An important factor, which can mediate the toxic action of DOX, especially in mitochondria, is the high affinity

binding of DOX to cardiolipin, an anionic phospholipid specific to the inner mitochondrial membrane, which has been recognized as an essential phospholipid in eukaryotic energy metabolism [128]. Cardiolipin, with its particular ability to interact with many proteins, is very important for mitochondrial structure and function. The toxicity of mitochondrial, mostly cardiolipin-bound, DOX is mediated by oxidative stress. Oxidative and nitrosative stress interfere with many aspects of cardiac function, inducing among others energetic imbalance, mitochondrial permeability transition and apoptosis, as well as activation of various related signaling pathways. The functional and structural changes in mitochondria that occur after DOX exposure suggest that ROS generation is a result of changes in the transcriptome rather than redox cycling of DOX [129].

In addition to oxidative stress, DOX may trigger other signaling cascades that may be related to the observed toxicity. HSF activation is one such consequence. HSPs are expressed in tissues by activation of the HSFs (HSF-1, HSF-2, HSF-3, and HSF-4), which are known to respond to various stresses. Upon oxidative stress, HSF-1 is trimerized and translocated to the nucleus to transcribe various HSPs. Thus HSP25 is an essential constitutive protein, but with either increased expression or decreased degradation due to the exerted stress, it causes a redox imbalance leading to tissue damage, especially in the heart [130]. Overexpression of HSP20 in the heart attenuates DOX-induced cardiac injury. The mechanism underlying its protection depends on the maintenance of Akt signaling cascades (Akt/BAD/caspase-3); and the attenuation of DOX-triggered oxidative stress, leading to inhibition of DOX-induced cardiomyocyte death and apoptosis [65].

ANTHs and Immunogenic cell death

The immune system plays a crucial role not only during oncogenesis and tumor progression, but also in the way established neoplastic lesions respond to therapy. Cytotoxic chemicals, such immunogenic chemotherapeutics including ANTHs can indeed elicit ICD. In the concept of ICD, the analysis of surface proteome changes in ANTH-treated tumor cells shows that ICD is associated with the ectopic exposure of the ER chaperone CRT. The ability of DOX to induce ICD was shown to depend on the induction of ER stress. The combined action of ROS and ER stress was shown to activate danger signaling pathways that help to traffic DAMPs to the extracellular space. ROS were proposed to be crucial because the immunogenicity of ICD was found to be diminished in the presence of antioxidants. Moreover, the simultaneous presence of ER stress and ROS production increased the number of different DAMPs emitted [131]. DAMPs include surface-exposed CRT, secreted ATP, and high mobility group protein B1 (HMGB1, also known as amphoterin). The extracellularly released HMGB1 induces intense inflammation, stimulating the production of proinflammatory cytokines such as TNF, IL-6, and IL-8 from neutrophils, macrophages, and monocytes [132]. In response to ANTH, CRT exposure occurs before the first morphological signs of apoptosis and before the translocation of phosphatidylserine from the inner to the outer area of the plasma membrane [133]. Interestingly, HMGB1 is a redox-sensitive protein.

Thus, the differential activity of HMGB1 in immunity, inflammation and cell death depends on the cellular redox status within tissues [134].

Treatment with ANTHs: incidences and risk factors

ANTHs such as DOX, epirubicin, and daunorubicin appear to be among the most active anticancer agents for the treatment of a large variety of solid tumors and hematological malignancies. Many chemotherapeutic drugs have detrimental effects on cardiovascular functions [135–138]. These agents and their cardiovascular side effects are summarized in Table 1.

Cardiac toxicity

Depending on the dose, the pharmacokinetics, and the type of ANTH used, myocardial cell loss or functional damage can occur. Morphological changes to the myocardium following ANTH treatment include myocardial cell loss by necrosis or apoptosis, the loss of both myofibrils and sarcoplasmic reticulum, and mitochondrial swelling [116,128]. DOX-induced cardiotoxicity may be divided into acute, subacute and late forms. Increases in levels of troponins I are indicative of cardiomyocyte injury, and brain natriuretic peptides (BNPs) and N-terminal prohormone of BNP might reflect increased myocardial stress. Acute DOX-induced cardiotoxicity occurs in up to 30% of patients. This cardiotoxicity starts within 24 h of the infusion and includes ECG abnormalities such as atypical ST changes, reduced QRS voltages, tachycardia, and supraventricular premature beats. Early toxicity develops months after the last chemotherapy dose and typically presents as new onset heart failure with left ventricular systolic dysfunction. The prevalence of left ventricular contractile dysfunction in patients with a cumulative DOX dose of approximately 430–600 mg/m² is about 50–60%, in whom a significant incidence of cardiac diseases are observed. The incidence of heart failure is nearly 2% with a cumulative dose of 300 mg/m² but rapidly increases to 20% at cumulative doses in excess of 550 mg/m² [139]. A chronic side effect of DOX is its dose-dependent cardiotoxicity. Subacute cardiotoxicity is rather rare, and appears several weeks or months (as late as 30 months) after the last dose of ANTH. The most frequent disease observed in patients is pericarditis; it is a condition in which the sac-like covering around the heart (pericardium) becomes inflamed. Moreover, patients treated at a younger age appear to be more vulnerable to ANTH-induced cardiotoxicity. An age of < 4 years at the time of exposure is associated with a significant risk of later cardiac dysfunction. The chronic form may not become evident until as many as 4–20 years after the last administration of DOX, and is associated with progressive myocardial dysfunction (dilated cardiomyopathy and congestive heart failure). Late reactions are seen years after presentation as new-onset cardiomyopathy often in patients who were treated for childhood neoplasms [140,141]. A population-based study of breast cancer survivors showed that women aged 66 to 70 years who received ANTH and had a follow-up of more than 10 years experienced higher rates of CHF than did women who received neither ANTH nor chemotherapy [142]. It is important to remember that the degree and progression

Table 1. Anticancer therapy and cardiovascular toxicity [135–138]^a

	Arrhythmia	Long QT	Myocardial ischemia	Thromboembolism	Systolic dysfunction	Hypertension
Anthracyclines (ANTHs)						
DOX	+++	NE	+	NE	+++	+
DOX (liposomal)	+	NE	++	NE	+	+
Epirubicin	+	NE	+	NE	+	+
Daunorubicin	++	NE	+	NE	+	+
Idarubicin	+++	NE	+	NE	++	+
Mitoxantrone	+++	NE	++	NE	++	++
Monoclonal antibody						
Trastuzumab (TRZ)	++	NE	+	++	+++	++
Bevacizumab	++	NE	++	+++	++	++
Cetuximab	++	NE	+	+	NE	++
Brentuzimab	+	NE	+	+	+	+
Ipilimumab	+	NE	+	+	NE	NE
Panitumumab	+	NE	++	++	NE	++
Pertuzumab	+	NE	+	+	++	+
Rituximab	+	NE	++	+++	+	++
Tyrosine kinase inhibitors						
Dasatinib	+++	++	++	++	++	++
Nilotinib	++	++	NE	+	++	+++
Vemurafenib	++	NE	++	++	+	++
Sorafenib	+	NE	++	++	++	+++
Sunitinib	+	+	++	++	+++	+++
Erlotinib	NE	NE	++	++	NE	NE
Gefitinib	NE	NE	++	++	NE	NE
Imatinib	NE	NE	+++	++	++	NE
Lapatinib	NE	+++	++	+	++	NE
Pazopanib	NE	NE	++	++	+	+++
Proteasome inhibitors						
Bortezomib	+	NE	+	+	+	+
Carfilzomib	++	NE	++	NE	+	+
Hormone therapy						
Tamoxifen	+	NE	++	++	++	++
Abiraterone	++	NE	++	NE	++	++
Anastrozole	NE	NE	++	++	NE	++
Exemestane	NE	NE	++	++	NE	++
Letrozole	NE	NE	+++	++	NE	++
Antimetabolite						
5-Fluorouracil	+++	NE	+++	NE	+	NE
Capecitabine	++	NE	++	++	NE	NE
Alkylating agent						
Cisplatin	NE	NE	NE	++	NE	NE
Cyclophosphamide	NE	NE	NE	+	NE	NE
Ifosfamide	NE	NE	NE	+	++	NE
Antimicrotubule agent						
Paclitaxel	++	NE	+	NE	+	+
Nab-paclitaxel	++	NE	NE	+	NE	+
Docetaxel	++	NE	++	NE	+	++

^a+++ represents >10%; ++, 1–10%; +, <1%; NE: not well established.

of ANTH-related toxicity differ among individuals, suggesting that the genetic predisposition and risk factors are involved [143].

Radiation therapy is frequently used in combination with chemotherapy and may worsen the cardiotoxic effects of ANTH. Experimentally, we demonstrated in rat hearts that the combination of DOX and cardiac irradiation could precipitate the unexpected expression of congestive heart failure. Oxidative lesions induced by irradiation and DOX

could represent one of the pathogenic factors of myocardial dysfunction [144].

Iron chelators as chemotherapeutic agents

Tumors possess altered iron homeostasis, which is mediated by the perturbed expression of iron-related proteins. Cancer cells have a higher uptake and utilization of iron by virtue of possessing significantly higher levels of transferrin receptor 1 than healthy cells [145]. Iron chelators have

also been studied as anticancer agents, because cancer cells have a higher requirement for iron than healthy cells due to their rapid rate of proliferation. Iron chelation has long been considered a promising strategy to limit cumulative, dose-dependent cardiac toxicity either by restoring cellular iron homeostasis or by removing redox-active iron, which may promote ANTH-induced oxidative stress. The development of several newer generations of cardioprotective iron chelators was a pharmacological intervention in response to the cardiotoxicity by DOX, which is an iron chelator with TOP-inhibitory and DNA damaging activity [146].

One iron chelator that has consistently shown cardioprotective ability *in vitro* and *in vivo* test systems is dexrazoxane (ICRF-187) (Figure 1). Dexrazoxane remains the only approved drug for the prevention of ANTH-induced cardiomyopathy. Dexrazoxane is a bisdioxopiperazine that is orally active as a prodrug. The mechanism of action of this drug in cardioprotection is that the relatively non-polar compound is taken up by cardiomyocytes, where it is then converted to its ring-opened hydrolysis product ADR-925, which is a diacid-diamide analog of EDTA. ICRF-187 (the ring closed form) is also a catalytic inhibitor of the nuclear enzyme TOP2. ADR-925 can rapidly displace iron from ANTH, suggesting that it has a stronger affinity for iron than does ANTH [147].

Several established and investigational iron chelators such as thiosemicarbazone derivatives inhibit TOPs. 3-Aminopyridine-2-carboxyaldehyde thiosemicarbazone (3-AP or triapine) reduces TOP1 activity. Among those known to chelate iron and target TOP2 α are dexrazoxane, (E)-N,N-dimethyl-2-(quinolin-2-ylmethylene)hydrazinecarbothioamide (TSC-24), and Dp44mT [148]. TSC-24 is considered a catalytic TOP2 α inhibitor due to a direct interaction with the ATPase domain of TOP2 α , which leads to a blockade of ATP hydrolysis [149]. The two mechanisms: higher levels of iron metabolism and elevated expression of TOPs may work completely independently to elicit the convergent goal of growth arrest and cell death. Another possibility is that dual targeting may accentuate TOP inhibitory action [148]. Many chemotherapeutic drugs as well as targeted chemotherapeutic agents have been tested as single agents or in combinations. Cancer cells acquire drug resistance via various mechanisms. HO-1 is a key enzyme exerting potent cytoprotection; HO-1 plays crucial roles in physiological iron homeostasis and antioxidant defense, and has anti-inflammatory and anti-apoptotic effects [14]. Recent studies show that inhibition of HO-1 induced the sensitization of human carcinoma cells to DOX. Inhibition of HO-1 could be a strategy to enhance the response of carcinoma to chemotherapeutic drugs [150].

ANTHs, immunity and cardiotoxicity

Stimulation of TLRs to activate the innate immune system has been a therapeutic strategy for some years. TLRs 3, 4, 7, 8, and 9 are all validated targets for cancer. The products that have been developed specifically to target TLRs in cancer therapy are the imidazoquinolines, imiquimod and resiquimod, which target TLR7. The dominant antitumoral mode of action of these agents is TLR7-mediated activation

of the central transcription of NF- κ B, leading to the induction of proinflammatory cytokines and IFN- α [151]. However, TLR7 agonists are often cardiotoxic at or above therapeutically-effective doses [152].

The objective of a recent study was to evaluate the therapeutic effect of TLR2 and TLR4 blockade on already established DOX-induced cardiomyopathy. The most significant findings of this study were that the blockade of TLR2 attenuated left ventricular dysfunction and fibrosis in DOX-triggered acute and chronic cardiomyopathy. This attenuation was strongly associated with reduced inflammation and reduced TLR2 endogenous agonist levels. By contrast, TLR4 inactivation aggravated DOX-induced cardiac injury and dysfunction, which was related to increased inflammation and decreased autophagy. This study provided direct evidence of the differential effects of TLR2 or TLR4 inhibition on DOX-induced acute and chronic cardiomyopathy [153].

Non-oligonucleotide small-molecule inhibitors of TLR9, such as AT791 (3-[4-(6-(3-(dimethylamino)propoxy)benzo[d]oxazol-2-yl)phenoxy]-N,N-dimethylpropan-1-amine)- and E6446 (6-[3-(pyrrolidin-1-yl)propoxy]-2-(4-(3-(pyrrolidin-1-yl)propoxy)phenyl)benzo[d]oxazole), are being developed. These compounds are orally bioavailable. AT791 and E6446 are typical of 'lysosomotropic' compounds in that they are lipophilic and contain weak base amines. Interestingly, TLR9 has been shown to influence myocardial function, and the cardioprotective effects due to TLR9 deficiency were associated with suppression of the TLR9 downstream pathway [154]. The severity of DOX-induced intestinal injury can also be reduced by using a TLR9 antagonist, which suggests a new therapeutic strategy for limiting DOX-induced intestinal inflammation [155].

Obesity and chemotherapy

Obesity is associated with a poor outcome in breast cancer patients treated with DOX-based chemotherapy [156,157]. The mechanism by which obese organisms display enhanced sensitivity to DOX-mediated toxicity is unknown. The dysregulation of adipocyte-derived hormones, adipocytokines, promotes the development of diverse obesity-linked diseases [158]. Plasma adiponectin levels are decreased in obese subjects. Adiponectin confers resistance to DOX-induced myocardial damage through activation of Akt signaling within cardiomyocytes. Adiponectin knockout mice showed exacerbated left ventricle contractile dysfunction following DOX injection, whereas exogenous adiponectin improved DOX-induced left ventricular dysfunction in wild type and adiponectin knockout mice [159]. It has also been demonstrated that metformin protects cardiomyocytes from DOX-induced damage and that the cardiac adiponectin system plays an important role in this protective action [160].

Evidence clearly supports a concept in which cancer cells reprogram adipocytes to cancer-associated adipocytes. Reprogrammed adipocytes produce growth-promoting cytokines and provide lipids and other metabolites to cancer cells, thus promoting uncontrolled tumor growth [161]. DOX treatment affects lipid and glucose metabolism. It increases serum total cholesterol, triglyceride, and LDL cholesterol levels. DOX inhibits adipogenesis in a

dose-dependent manner and downregulates the expression of peroxisome proliferator-activated receptor, PPAR γ , leading to the prevention of bodyweight gain through the inhibition of adipogenesis. Activation of PPAR γ using a PPAR γ agonist may be useful in controlling bodyweight loss [162].

TRZ and cardiotoxicity

Monoclonal antibody (mAb) therapy

In the past 15 years, 12 therapeutic antibodies have obtained FDA approval for hematological malignancies as well as solid tumors [163]. mAbs are an important group of targeted therapeutics which are directed against transmembrane proteins with extracellular domains. Several mAbs have entered clinical practice. Notable examples include TRZ (Herceptin®), lapatinib (Tykerb®), pertuzumab (Omnitarg®), panitumumab (Vectibix®), rituximab (Mabthera®/Rituxan®), and cetuximab (Erbix®) [164]. Four HER2-targeted therapies have been approved for HER2-positive breast cancer: two antibodies (TRZ and pertuzumab), an antibody–drug conjugate (ado-TRZ emtansine), and a small-molecule kinase inhibitor (lapatinib). TRZ emtansine (Kadcyla™) is an antibody–drug conjugate consisting of TRZ covalently linked to the highly-potent microtubule inhibitory agent DM1 (a cytotoxic derivative of maytansine) via a stable thioether linker [165].

TRZ complementarity-determining region amino acids complement and bind to amino acids present on domain IV of the HER2 ectodomain. Its functions are divided into those mediated by Fab (fragment, antigen binding) or Fc (humanized fragment) regions. The Fab region contains the antigen-binding sites of the antibody, whereas the Fc region contains the binding sites for Fc γ receptors (RIII) present on immune cells, platelets, hepatocytes, and endothelial cells [166]. The interaction between the Fab region of TRZ and a peptide fragment from HER2 was investigated with molecular dynamics simulations. The interaction energies of the mutated peptides indicated that TRZ binds to ligand through electrostatic and hydrophobic interactions [167]. The second HER2-targeted mAb, pertuzumab, binds to subdomain II of HER2 extracellular domain (Figures 4 and 5).

Among the immune cells, natural killer (NK) cells constitute a group of normal lymphocytes that induce innate immune responses towards tumor and virus-infected cells. Treatment strategies to manipulate human NK cell functions have involved immunotherapies to treat cancer. Several approaches have been designed to enhance the NK cell-mediated ADCC activity through antibody engineering [168]. ADCC is considered a major mode of action of many therapeutic mAbs, including treatments for cancer [169]. Some of the clinically approved therapeutic antibodies to treat cancer, such as TRZ, rituximab (anti-CD20 mAb), cetuximab (anti-EGFR mAb), and mogamulizumab (anti-CCR4 mAb), are considered to function at least partially through triggering NK cell-mediated ADCC activity. TRZ, which can be used to treat HER2/neu-positive breast cancer patients, mediates abrogation of tumor cell signaling and ADCC [102]. HER2 is found to be overexpressed in 25–30% of breast cancer patients (breast and gastric cancers). TRZ is currently recommended as the first-line

treatment for patients with metastatic HER2⁺ tumors, either as a single agent (limited group of patients) or in combination with endocrine therapy or chemotherapy. The use of TRZ may be limited by the development of drug intolerance, manifesting as cardiac dysfunction, for example [2,170].

TRZ: signaling, gene expression, and cardiac dysfunction.

Clinical trials of TRZ have reported heart failure in 1.7–4.1% of subjects, and reduced left ventricular ejection fraction in 7.1–18.6% of subjects treated with adjuvant chemotherapy and TRZ [171]. Risk factors specific for TRZ-associated cardiotoxicity have not been clearly established, although the majority of patients had other risk factors for cardiac dysfunction. Analyses of the potential risk factors, including age, weight, hypertension, cumulative dose, and HER2 expression level, have revealed that only age and concurrent DOX therapy were significantly associated with an increased risk of cardiac disease [171]. Because the cardiotoxicity caused by this agent was reversible upon discontinuation of treatment or initiation of appropriate cardiovascular therapy, a mechanism not related to loss of the terminally differentiated cardiomyocytes was suggested. The clinical cardiotoxicity of TRZ, together with the dilated cardiomyopathy observed in NRG-1/ErbB-deficient mice, suggests a prominent role of NRG-1 in the pathogenesis of chronic heart failure [172]. Various strategies have been developed to reduce the cardiotoxicity of TRZ without significantly compromising its therapeutic efficacy. These include optimization of chemotherapeutic combinations, shortening treatment duration, and careful monitoring of patients. Different mechanisms are proposed for TRZ cardiotoxicity. It is likely that TRZ-induced cardiotoxicity results from interference with the action of NRG. NRG-1 β activates phosphorylation of ErbB2 and ErbB4 receptors, which are expressed in cardiac myocytes of the adult rat. NRG-1/ErbB activity interacts with myocardial metabolism, thus improving mitochondrial function. Thus, the inhibition of ErbB2 signaling by TRZ in patients receiving DOX may interfere with the protective effects of NRG on the ANTH-damaged myocardium. This may account for the increased clinical cardiotoxicity observed with concurrent and sequential administration of ANTH and TRZ [2]. Clearly, the most-effective means to limit DOX/TRZ-induced cardiotoxicity is to modulate the dosages and increase the time between DOX and TRZ treatment initiation.

Interestingly, recent results show that, in mice, TRZ treatment induces major effects on the expression of myocardial genes involved in myocardial functions, adaptation to stress, and DNA repair. These genetic changes are associated with increased myocardial oxidative and nitrosative stress, and activate apoptotic pathways, leading to elevated serum troponin-I and cardiac myosin light chain-1 (cMLC1) levels [173]. A particularly important mechanism for TRZ-mediated cardiovascular disease appears to be via specific signaling pathways including Notch and NF- κ B.

The relationships between Notch factors and ErbB2 are complex, and recently it has been demonstrated that Notch-1 is a novel target in TRZ-resistant breast cancer,

suggesting that combined inhibition of Notch and ErbB2 signaling pathways may be beneficial in the treatment of recurrent TRZ-resistant disease [174]. Notch signaling regulates cardiovascular development and homeostasis, and plays a role in regulating cardiac hypertrophy, cardiomyopathy, and heart failure [175,176]. TRZ treatment was able to effectively target tumor-initiating cells of ErbB2-positive breast cancer cell lines. However, Notch-1 has been implicated not only in the self-renewal of these tumor-initiating cells but also in TRZ resistance [177]. In this context, results from clinical studies of TRZ and animal studies using transgenic mouse models or pharmacological approaches have demonstrated that major kinases of the HER2–PI3K pathway are important for regulating cardiac physiological function [178]. Phosphorylation of Akt represents PI3K pathway activation. This activation is regulated by different proteins such as survivin. Survivin modulates integrated cellular networks that are essential for tumor cell proliferation and viability. Survivin has been identified as a member of the IAP family, which is undetectable in normal cells but overexpressed in several human cancers [179]. Survivin activity can be regulated by ErbB2 through the PI3K–Akt pathway. The importance of the PI3K/Akt/survivin pathway has been reported in lung and colon cancer [180]. HER2 therapy has been successful in many cases, but patients have a tendency to develop resistance to the inhibitory agents [181]. Increased ErbB2 expression has been associated with drug resistance in cancer cells [182]. In this field, the role of oxidative stress has been evoked. In the Calu-3 cell line, it has been demonstrated that TRZ treatment was associated with an increase in cellular ROS production, glutathione depletion, and a decrease in the activities of SOD and catalase enzymes. In addition, impaired intracellular antioxidant/oxidant balance contributes to TRZ-mediated cell death [183]. There is crosstalk between the amount of HSPs, the antioxidant/oxidant balance, and intracellular redox homeostasis. HSP90 is able to possess reactive cysteines and was able to reduce cytochrome *c*, suggesting that this chaperone plays a role in modulating the redox status in resting and apoptotic cells [184]. HSP90 is able to buffer the effects of oxidative stress; HSP90 activity counteracts the effects of oxidative stress on enzyme functions [185]. In cancer, however, the chaperoning activity of HSP90 is often exploited by cancer cells to confer aberrant proliferative, survival, and/or metastatic potential. Pharmacologic blockade of HSP90 is an innovative approach in the development of new antineoplastic agents [186]. The combination of different HSP90 inhibitors with TRZ could also be effective, as is the case in HER2-positive breast cancer patients [187,188].

DOX–TRZ: synergic effects on cardiotoxicity

The addition of TRZ to adjuvant DOX chemotherapy has reduced the risk for breast cancer recurrence by 50% and mortality by 30% in ErbB2-positive women. However, the major limitation of this therapeutic regimen is the onset of serious cardiac side-effects. The combination of DOX and TRZ therapy induced a synergistic detrimental cardiotoxic effect [189]. Subsequent TRZ adjuvant trials were designed to include prospective evaluations of cardiac effects:

NSABP B-31 (National Surgical Adjuvant Breast and Bowel Project), NCCTG N9831 (North Central Cancer Treatment Group), BCIRG 006 (Breast Cancer International Research Group), FinHer (Finland Herceptin trial), and the Herceptin Adjuvant trial (HERA). In the HERA trial, long-term evaluation at 8 year median follow-up validates the low incidence of cardiac events for TRZ given sequentially after chemotherapy and radiotherapy. Importantly, cardiac events were reversible in the majority of patients [190]. Better understanding of the molecular mechanisms responsible for the synergistic TRZ-induced cardiac injury associated with ANTH-treatment may be important for novel protective or preventive therapeutic strategies. As we reported, TRZ cardiotoxicity is due to the deregulation of ErbB2/4–PI3K signaling, which is essential to maintain cellular homeostasis. As developed previously, one of the mechanisms by which DOX causes deleterious cardiac structural and functional changes involves oxidative stress. Recently, it has been demonstrated that, in mice, TRZ alone induced only a transient increase in myocardial 3-nitrotyrosine staining, a biomarker of nitrogen free radical species. DOX treatment induced sustained oxidative and nitrosative stress, which was exacerbated by DOX and TRZ in combination [191] (Figure 6).

The precise mechanisms underlying DOX/TRZ-induced cardiotoxicity remain incompletely understood. A significant number of adverse effects occur in patients without obvious risk factors. Some patients develop intense DOX-induced cardiotoxicity at low doses, whereas other patients tolerate very high doses without any myocardial disease. Concerning treatment with TRZ, there is no relationship between the cumulative dose and the probability of developing cardiotoxicity. Recently, some studies investigated candidate-gene approaches to genotyping risk for anticancer drug-induced cardiotoxicity. Variability in susceptibility to myocardial damage is incompletely explained by clinical factors, and the evidence demonstrates a genetic predisposition [192]. Early changes in biomarkers may be useful in predicting adverse cardiovascular outcomes with DOX and TRZ. Concerning TRZ treatment and its cardiotoxicity, pharmacogenetics is an essential approach. It determines whether there is a correlation between genetic polymorphism, such as in *HER2*, and the response to TRZ treatment or the development of TRZ-associated cardiotoxicity. Most reported polymorphisms affecting the efficacy of anticancer treatment are single-nucleotide polymorphisms [193]. Crosstalk between the estrogen receptor pathway and the PI3K pathway is thought to be involved in resistance to TRZ-containing chemotherapy [194].

When TRZ is used in association with DOX, the development of heart failure is potentiated (Figure 6). This could be related to the inherent capacity of DOX to increase oxidative stress. The use of angiotensin II type 1 (AT1)-inhibitors in patients with troponin elevation during chemotherapy may be an effective tool to prevent left ventricular ejection fraction reduction [195]. In experimental studies, we demonstrated that DOX induced alterations in cardiac function, inflammation, and plasma oxidative stress whereas tissue oxidative stress and cardiac kinin

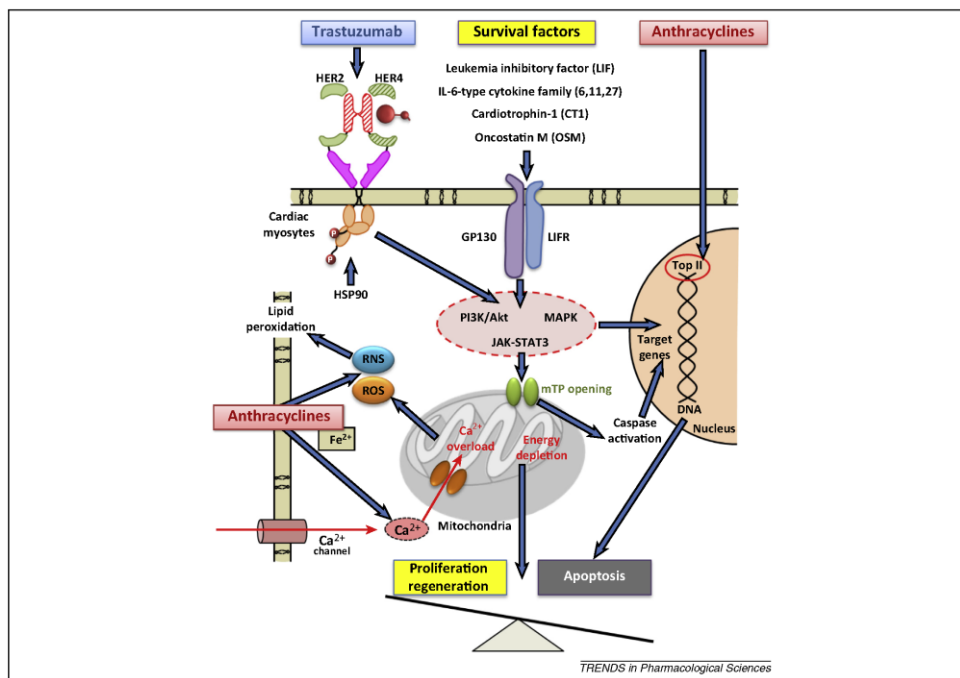


Figure 6. Molecular mechanisms and novel therapeutic approaches in cardioprotection in patients treated with ANTH and trastuzumab. Leukemia inhibitory factor (LIF) may either stimulate proliferation or induce differentiation depending upon the cell type and its stage of development. LIF is a member of the IL-6 family, which includes the IL-6-type cytokine family (IL-6, -11, -27), cardiotrophin-1 (CT1), and oncostatin M (OSM). All IL-6-type cytokines assign the common signal transducer gp130. LIF evokes genomic and non-genomic events to protect cardiac myocytes. LIF induces dimerization of gp130 and LIFR, activating signaling pathways (PI3K/Akt, MAPK, JAK-STAT3). These actions are linked to cellular protection, cell regeneration, and/or apoptosis.

receptor expression were not modified. AT1 inhibition did not improve cardiac performance but it modulated kinin receptor expression and enhanced antioxidant defenses [11]. In the clinical field, several studies have evaluated the efficacy of cardiac medications in preventing or reversing cancer therapy-induced cardiotoxicity with angiotensin-converting enzyme inhibitors or angiotensin II receptor blocker. Studies with these drugs have shown their utility in preventing and reversing chemotherapy-induced left ventricular dysfunction [196].

It is well established that the signaling pathway mediated by the AT1 receptor plays an important role in DOX- or TRZ-induced cardiac impairment, suggesting that an AT1 receptor blocker (ARB) might be used to prevent ANTH-induced cardiomyopathy. A large body of evidence has clarified the role of AT1 in mediating the development of cardiac hypertrophy and left ventricular remodeling after myocardial infarction or acute ANTH exposure. DOX induces myofibril loss, increases the number of apoptotic cells, and significantly impairs cardiac function in control mice, but not in AT1 knockout mice or in animals treated with an ARB. It appears that the AT1-mediated signaling pathway plays an important role in DOX-induced cardiac failure [197].

More recently, an indirect relationship has been considered between the renin-angiotensin system and TRZ-induced cardiac dysfunction. TRZ binds to HER2 with high affinity, thereby eliminating its ability to dimerize with other HER receptors. By blocking HER2 signaling, cardiomyocytes are unable to activate the cell survival pathways associated with excess ROS. Therefore, blockade of HER2 allows the accumulation of ROS within cardiomyocytes, which leads to the development of cardiac dysfunction associated with cellular apoptosis. Combination therapy with DOX and TRZ increases the formation of ROS, thus amplifying cardiac dysfunction. The increased stress on the heart leads to the upregulation of circulating ANG II, which in turn contributes to the detrimental effects on the heart [170]. It has been also demonstrated that the pleiotropic biochemical and cellular effects of TRZ and DOX induce ER stress related to oxidative stress [198,199].

Concluding remarks

It is now clearly demonstrated that several chemotherapeutic agents, combined or not with radiation therapy, are associated with an increased risk of myocardial disease. The best treatment for chemotherapy-induced

cardiotoxicity is prevention. Cardiospecific biomarkers have proved to be a valid diagnostic tool for the early identification and monitoring of cardiotoxicity. Our understanding of the mechanisms by which these therapies affect the heart is crucial for improving drug design and finding alternative therapies to protect patients predisposed to cardiovascular disease. Ideally, the goal of new therapies should be to improve the management of cancer through the specific targeting of malignant cells and fewer adverse cardiac side-effects. New therapies include tyrosine kinase inhibitors, antibody–chemotherapy conjugates, HSP inhibitors, and antibodies that interfere with the formation of ErbB2–ErbB3 dimers [106]. Recent bioinformatics and proteomic analyses have uncovered several previously unrecognized roles for specific proteins in regulating cell physiology under normal and stressed conditions. The optimal development and application of HSP90-targeted therapies will depend on synthesizing information gained from a careful genetic analysis of primary and metastatic tumors. BIIB021 is the first oral, synthetic, HSP90 inhibitor that showed activity when administered at nM concentrations to subjects with advanced solid tumors [200,201].

The major hypothesis for anticancer drug-induced cardiotoxicity attributes the toxicity to several causes involving the cellular basis for growth, hypertrophy, and failure of the human heart. In this context, the future of pharmacological regeneration may lie in the local delivery of molecules targeting specific growth and differentiation pathways [202]. Chemopreventive strategies could represent another way to reduce cardiotoxicity. A detailed history of patients that focuses on cardiovascular risk factors (diabetes, obesity, pre-existing cardiovascular disorders) and mediastinal irradiation is required [203]. New approaches to personalized treatment for cancer that involve molecular screening for clinically relevant genomic alterations and genotype-targeted treatments are emerging.

In this arena of clinical oncology, the next decade should witness the development of novel therapeutic approaches to cardioprotection in patients treated with ANTH and TRZ [204]. Evidence in recent years has clearly established the beneficial action of cytokine leukemia inhibitory factor (LIF) in preventing injury to the myocardium associated with various drugs (Figure 6). LIF is a member of the IL-6-type cytokine family, which includes IL-6, IL-11, IL-27, LIF, ciliary neurotrophic factor (CNTF), cardiotropin-1 (CT-1), oncostatin M (OSM), and cardiotrophin-like cytokine-1/novel neurotrophin-1/B cell stimulating factor-3 (CLC-1/NNT-1/BSF-3). Current evidence suggests that CT-1, a 201 amino acid protein, plays an important role in the regulation of the cardiovascular system. CT-1 has a large number of functions that sometimes have opposite effects. In fact, it can promote cardiac cell survival but can also cause cardiac hypertrophy and ventricular remodeling [205]. LIF signals through a shared gp130 receptor [206]. For LIF, signal induction occurs when this cytokine binds to the LIF receptor and evokes its dimerization with gp130. LIF activates different pathways in cardiac myocytes such as (i) JAK–STAT and MAPK, and (ii) PI3K/Akt.

The mechanism has been ascribed to inhibition of opening of the mPTP in response to increases in intracellular Ca^{2+} and ROS [207]. Evidence indicates that the gp130-mediated signaling networks play important roles in the progression of multiple types of cancer. Inhibition of gp130 activity offers a potential and promising approach to cancer therapy.

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1. Métabolisme du fer dans le système cardiovasculaire

Le fer présente de multiples fonctions biologiques, notamment dans la formation de l'hémoglobine, de la myoglobine et de nombreuses enzymes. Le métabolisme du fer fait appel à des processus de régulation et de transport extrêmement complexes. En effet, l'homéostasie du fer nécessite une communication entre les sites d'absorption, de stockage et d'utilisation de cet élément. En cas de défaillance de ces mécanismes, une carence ou une surcharge en fer peuvent apparaître. Les conséquences de ces perturbations de l'homéostasie ferrique ont fait l'objet d'une attention croissante de la communauté scientifique ces dernières années. Ces interactions et notamment le rôle de l'hepcidine comme cible thérapeutique ont été détaillés dans la revue de la littérature suivante, que nous avons publié en 2014 dans *Pharmacology and Therapeutics* (84). Le fer est le seul micronutriment connu à avoir une hormone de régulation, l'hepcidine, qui répond à la fois à l'état nutritionnel et aux infections. L'hepcidine agit pour bloquer à la fois l'absorption du fer dans l'intestin et le relargage du fer à partir de macrophages par le biais d'un mécanisme commun. La découverte du rôle hormonal de régulation du fer de l'hepcidine, puis l'élucidation de son mécanisme d'action, ont conduit à une meilleure compréhension de la physiopathologie des pathologies humaines liées au fer. Les régulateurs positifs de l'hepcidine, qui conduisent à la réduction de la ferroportine et une diminution de la disponibilité du fer, incluent une augmentation du fer sérique et des cytokines inflammatoires circulants. Il apparaît que la mesure des taux d'hepcidine pourrait être utile dans le diagnostic de plusieurs pathologies cardiovasculaires associées à des hémopathies. Les pathologies engendrant une surcharge en fer sont généralement insidieuses et induisent des lésions d'organes progressives et irréversibles, avant que les symptômes cliniques n'apparaissent. En outre, une meilleure compréhension de la relation entre la structure et la fonction de hepcidine et le complexe hepcidine-ferroportine pourrait conduire à la conception d'antagonistes et agonistes de l'hepcidine en thérapie humaine. Le développement de la recherche dans ce domaine offre un nouveau potentiel clinique en termes de diagnostic et de thérapies ciblées.



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The iron-regulatory hormone hepcidin: A possible therapeutic target?



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ABSTRACT

The maintenance of stable extracellular and intracellular iron concentrations requires the coordinated regulation of iron transport into plasma. Iron is a fundamental cofactor for several enzymes involved in oxidation–reduction reactions. The redox ability of iron can lead to the production of oxygen free radicals, which can damage various cellular components. Therefore, the appropriate regulation of systemic iron homeostasis is decisive in vital processes. Hepcidin has emerged as the central regulatory molecule of systemic iron homeostasis. It is synthesized in hepatocytes and in other cells and released into the circulation. It inhibits the release of iron from enterocytes of the duodenum and from macrophages by binding to the iron exporter protein, ferroportin (FPN). FPN is a transmembrane protein responsible for iron export from cells into the plasma. Hepcidin is internalized with FPN and both are degraded in lysosomes. The hepcidin–FPN axis is the principal regulator of extracellular iron homeostasis in health and disease. Its manipulation via agonists and antagonists is an attractive and novel therapeutic strategy. Hepcidin agonists include compounds that mimic the activity of hepcidin and agents that increase the production of hepcidin by targeting hepcidin-regulatory molecules. The inhibition of hepcidin could be a potentially attractive therapeutic strategy in patients suffering from anaemia or chronic inflammation. In this review, we will summarize the role of hepcidin in iron homeostasis and its contribution to the pathophysiology of inflammation and iron disorders. We will examine emerging new strategies that modulate hepcidin metabolism.

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Abbreviations: AA, amino-acid; ABCA1, ATP binding cassette transporter A1; ABCB, ATP binding cassette (ABC) transporter; AKI, acute kidney injury; AMI, acute myocardial infarction; ARE, antioxidant response element; ATP, adenosine triphosphate; CHD, coronary heart disease; CKD, chronic kidney disease; CHF, chronic heart failure; CO, carbon monoxide; COX, cyclooxygenase; CPB, cardiopulmonary bypass; CREBH, cyclic AMP response element-binding protein-H; CRP, C-reactive protein; DMT1, divalent metal transporter 1; EAM, experimental autoimmune myocarditis; EPO, erythropoietin; ER, endoplasmic reticulum; ESAs, erythropoietin-stimulating agents; FPN, ferroportin; GDF-15, growth differentiation factor-15; hHepc, human hepcidin; HIF, hypoxia-inducible factor; HJV, haemojuvelin; HMOB1, high mobility group protein B1; HO, haem oxygenase; H₂S, hydrogen sulphide; HSP, heat shock protein; IL, interleukin; IREs, iron-responsive elements; IRPs, iron-regulatory proteins; JAK, Janus kinase; Keap, Kelch-like ECH-associated protein 1; LPS, lipopolysaccharide; Lrp1, lipoprotein receptor-related protein-1; α 2M, α 2-macroglobulin; MAL, MyD88 adapter-like; NO, nitric oxide; NOS, nitric oxide synthase; NOX, nicotinamide adenine dinucleotide phosphate oxidase; Nrf2, nuclear factor erythroid-related factor 2; PEG, polyethylene glycol; RNS, reactive nitrogen and oxygen species; RNS, reactive nitrogen species; ROS, reactive oxygen species; sHJV-Fc, soluble HJV-Fc fusion protein; SOD, superoxide dismutase; STAT, signal transducer and activator of transcription; SR, sarcoplasmic reticulum; Tf, transferrin; TIR, TIR receptors; TLRs, toll-like receptors; TPP, thiamine pyrophosphate; TRAM, TLR4–TRIF-related adapter molecule; TTFD, thiamine tetrahydrofurfuryl disulphide.

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1. Introduction

Iron is a fundamental cofactor for several enzymes involved in oxidation–reduction reactions due to its ability to exist in two ionic forms: ferrous ($\text{Fe} + 2$) and ferric ($\text{Fe} + 3$) iron. However, the redox ability of iron can lead to the production of oxygen free radicals, which can damage various cellular components. For this reason, iron levels in tissues must be tightly regulated (Ganz, 2013). Various molecules are involved in iron uptake and storage by hepatocytes and its export from hepatocytes, and systems describing the iron cycle have evolved. The discovery of the iron-regulating role of the hormone hepcidin, followed by the elucidation of its mechanism of action has led to better understanding of the physiopathology of human iron disorders (Munoz-Bravo et al., 2013; Waldvogel-Abramowski et al., 2014) and offers new clinical potential in terms of diagnosis and therapy. Hepcidin has emerged as the central regulatory molecule of systemic iron homeostasis. Knowledge on how hepcidin exerts its regulatory function and on the molecular processes that regulate hepcidin production is largely based on animal and in vitro studies. Hepcidin is a peptide secreted predominantly from hepatocytes. It down-regulates ferroportin, the only known iron exporter, and therefore inhibits iron efflux from duodenal enterocytes, macrophages and hepatocytes into the bloodstream (Ganz & Nemeth, 2012). Hepcidin expression is regulated positively by body iron load. Although the underlying mechanism of iron-regulated hepcidin expression has not been fully elucidated, several proteins have been identified that participate in this process. In this review, we will summarize the role of hepcidin in iron homeostasis and its contribution to the pathophysiology of inflammation and iron disorders. We will examine emerging new strategies to modulate hepcidin metabolism. The therapeutic manipulation of hepcidin activity may become an important approach in cardiovascular and metabolic disorders.

2. Systemic iron metabolism and the importance of iron storage

2.1. Iron distribution

The total amount of iron in a 70-kg adult is approximately 4 g, of which two thirds is the iron in red blood cells and 300 mg is in the myoglobin of muscles. The majority of this iron comes from the recycling of senescent erythrocytes by macrophages of the reticulo-endothelial system (about 20 mg/day) (Gudjoncik et al., 2014). Most of the iron in plasma is directed to the bone marrow for erythropoiesis. More than 2 million new erythrocytes are produced every second by the bone marrow, requiring a daily supply of at least 20–30 mg of iron. Only 1 to 2 mg of the daily iron supply is derived from intestinal absorption, which, under a steady state, is sufficient only to replace insensible iron loss. Significant amounts of iron are also present in macrophages (up to 600 mg) whereas excess body iron (~1 g) is stored in the liver. Each erythrocyte contains a billion atoms of iron; at normal rates of turnover, this concentration corresponds to the incorporation of 2×10^{20} atoms of iron per day. Consequently, anaemia is the cardinal sign of iron deficiency. The best characterized syndrome of iron overload is hereditary haemochromatosis (Vujic, 2014). The acquisition, transport, utilization and storage of iron are tightly controlled to meet physiological needs

and prevent excessive accumulation of the metal within cells. Mammals utilize distinct mechanisms to regulate iron homeostasis at the systemic and cellular levels (Lawen & Lane, 2013).

2.2. Proteins that exert crucial functions in the maintenance of systemic iron homeostasis

2.2.1. A range of regulatory mechanisms: general regulation

Several proteins, such as transferrin, ferritin, hemosiderin, hepcidin and ferroportin, exert crucial functions in the maintenance of systemic iron homeostasis. Macrophages play an important role in executing the regulatory events that lead to changes in systemic iron levels (Gammella et al., 2014). Schematically, the main site of iron absorption is the small intestine, but most iron is recycled by the monocyte–macrophage system via phagocytosis of senescent erythrocytes. In the circulation, iron is usually bound to transferrin (Tf), and most of the Tf-bound iron is utilized for bone marrow erythropoiesis (Gudjoncik et al., 2014). Within cells, iron is stored in the proteins ferritin or hemosiderin. Iron is the only micronutrient known to have a regulatory hormone, hepcidin, which responds to both nutrient status and infections. Hepcidin is mainly synthesized in the liver. It is a negative regulator and its production is increased during iron overload and inflammation. Intracellular iron is released into the circulation via ferroportin (FP). The iron is donated to Tf and reutilized for bone marrow erythropoiesis. Hepcidin binds to the iron exporter FP and leads to its degradation, thereby inhibiting intestinal iron absorption, cellular export and reticulo-endothelial iron release (Munoz et al., 2011).

2.2.2. Role of ferritin

Multiple physiological processes are involved in maintaining iron homeostasis. These include iron storage at the intracellular and extracellular levels. Ferritin is the major iron-storage protein. Typical ferritins are composed of 24 subunits, which fold into a 4-helix bundle and form an almost spherical protein shell. There is a strong equilibrium between ferritin-bound iron (Fe^{3+}) and the labile iron pool in cells (Fe^{2+}), by which ferritin prevents the formation of reactive oxygen species mediated by the Fenton reaction (Alkhateeb & Connor, 2013). Various cell types contain a transient cytosolic pool of iron, presumably bound to low-molecular-mass intracellular chelates, such as citrate, various peptides, ATP, AMP or pyrophosphate. This labile iron pool is redox-active and its concentration is determined by the rates of iron uptake, utilization for incorporation into iron enzymes, storage in ferritin and export from the cell (Cabantchik, 2014).

There are two functionally and genetically distinct ferritin subunits: L-ferritin and H-ferritin (also known as light-chain and heavy-chain ferritin). Ferritin is found in the cytoplasm, nucleus and mitochondria of cells. Serum ferritin has been thought to reflect iron stores in the body and to increase as a secreted by-product of intracellular ferritin synthesis. Cellular iron is stored primarily in the cytoplasm, but organelles such as mitochondria are the main users of metabolically active iron (MacKenzie et al., 2008).

The regulation of ferritin synthesis by iron is mainly due to post-transcriptional regulation through the binding of IRP1 and IRP2 to iron-responsive elements (IRE) located in the 5' UTR of ferritin mRNA. Both IRP1 and IRP2 are expressed ubiquitously in most tissues. In

addition to iron-mediated regulation, oxidative stress is able to modulate the activity of IRP1 and IRP2. This process is regulated at the transcriptional level through an antioxidant-responsive element (ARE) (Tsuiji, 2005). The role of nitric oxide (NO) in the regulation of the IRP/IRE system is well established (Hentze et al., 2004). NO induces the expression of FPN, the transmembrane protein responsible for iron export from macrophages, and is associated with activating the transcription factor nuclear erythroid 2-related factor-2 (Nrf2) (Nairz et al., 2013). Given the established regulatory circuits between nitric oxide synthase (NOS) activity and iron homeostasis, it has been hypothesized that iron homeostasis and NO biology are interconnected. Recent studies showed that haemoglobin α haem iron in the oxygenated Fe^{2+} state and NOS are in a macromolecular complex and can form a direct protein–protein interaction (Straub et al., 2012).

2.2.3. Role of transferrin

Cellular iron homeostasis is accompanied by the coordinated regulated expression of ferritin and other proteins including Tf. Cells have transferrin receptors (TfR) that mediate iron metabolism. Iron is imported through the endocytosis of Fe^{3+} -loaded Tf that interacts with its TfRs (TfR1 and TfR2) in a tightly regulated feedback loop controlled at the genetic and protein level. Tf is a monomeric protein of 76–81 kDa comprising two structurally similar lobes (termed N- and C-lobes), each of which contains a single iron-binding site. Plasma Tf is a powerful chelator, capable of binding iron tightly but reversibly. Iron chelation by Tf serves to maintain Fe^{3+} in a soluble form in a redox-inert state, thus preventing the generation of toxic free radicals (Gkouvatsos et al., 2012). TfR1 (also known as CD71) is ubiquitously expressed at low levels in most normal human tissues. TfR2 is a protein that is highly homologous to TfR1 but whose expression is largely restricted to hepatocytes. Serving as the main port of entry for iron bound Tf into cells, TfR1 is a type II receptor that resides on the outer cell membrane. The Tf–TfR complex is internalized via clathrin-coated pits and then metabolized in the lysosome (Daniels et al., 2012). Iron homeostasis is controlled by Tf as well as in other ways. In this context, a relationship exists between TfRs and peptides such as hepcidin, which appears to be involved in the final common pathway towards which the components of the regulatory network converge to control tissue iron metabolism.

A decade ago, hepcidin, an antimicrobial peptide with iron-regulatory properties, was discovered and found to play a significant role in iron regulation in health and disease. Hepcidin is predominantly synthesized in hepatocytes, secreted from hepatocytes and excreted through the kidney. In iron deficiency or in haemorrhagic or haemolytic anaemia, the production of hepcidin in hepatocytes decreases. When this happens, both iron absorption in the duodenum and the release of iron from stores are greatly increased. In patients with anaemia or chronic disease, hepcidin is over-expressed (Polin et al., 2013). In the following chapters of this review, we will describe the structures and functions of hepcidin and examine emerging new pharmacological strategies that modulate hepcidin metabolism.

3. Diseases associated with iron deficiency or iron overload

3.1. Iron deficiency

The symptoms and signs of iron deficiency are partly explained by the presence of anaemia. Iron deficiency will result from any condition in which dietary iron intake does not meet the body's demands; for this reason, rapidly growing children and premenopausal women are at the highest risk. Iron deficiency caused by dietary insufficiency is usually secondary to intestinal blood loss. Congenital and acquired abnormalities of the intestinal epithelium can also result in iron deficiency. The combination of mild-to-moderate anaemia and hypoferritaemia is often seen in chronic infections, inflammatory disorders, haematological malignancies, and some solid tumours. Inflammation anaemia has

also been called the “anaemia of chronic disease,” (Corwin & Krantz, 2000). The influence of iron deficiency was underestimated especially in terms of worsening of cardiovascular diseases and of developing anaemia (Ebner & von Haehling, 2013). Approximately one third of patients with congestive heart failure (CHF) and 10% to 20% of those with coronary heart disease (CHD) also have anaemia. Anaemia is associated with more symptoms, a greater hospitalization rate and increased mortality rates in patients with CHF and CHD (Kansagara et al., 2013).

3.2. Iron overload: haemochromatosis

Iron overload leads to excessive iron deposition in a wide variety of tissues. The rapid and massive influx of iron into the plasma can cause an early-onset organ disorder called juvenile haemochromatosis, which includes heart failure and endocrine insufficiency. Primary (hereditary) haemochromatosis and secondary iron overload (hemosiderosis) is reaching epidemic levels worldwide. In genetic haemochromatosis and in β -thalassaemia, iron overload is the primary cause of liver cirrhosis, diabetes, and heart failure. Over 85% of all cases of haemochromatosis are due to mutations in the haemochromatosis protein, which is involved in iron metabolism. Human haemochromatosis protein also known as the HFE protein is a protein encoded by the HFE gene (Moyer et al., 2011). The cardiac and endocrine systems are particularly susceptible to rapid iron loading. In cells, iron induces an increase in oxidative stress and limits high-energy electron transport required for mitochondrial respiration (Murphy & Oudit, 2010). Four meta-analyses of the studies examining the association between four HFE gene variants and the risk of coronary heart diseases were recently conducted. HFE gene mutations were shown to be associated with iron overload and cardiovascular diseases (Lian et al., 2013).

The management of iron overload and the treatment of iron toxicity by chelation in patients with acquired iron overload (e.g. transfusion-dependent anaemia) have been shown to reduce iron burden and improve survival. Iron chelators are best known for their ability to sequester iron from the cell or organism and to function as an antioxidant by preventing the Fenton reaction. Three iron chelating agents: Deferoxamine, Deferiprone and Deferasirox are currently available (Munoz et al., 2011). Other orally active iron chelators are currently under development and one, FBS0701, is in a clinical trial. FBS0701 has been shown to bind Fe^{3+} with very high affinity and selectivity and has demonstrated a no-observable-adverse-effect level at least 4 times greater than that obtained with deferasirox suggesting a favourable clinical safety profile (Ma et al., 2012; Neufeld et al., 2012).

4. The liver: the main regulator of iron metabolism and producer of iron-regulatory proteins (Fig. 1)

The liver is the central regulator of systemic iron balance. Hepatocytes not only store iron, but also play a crucial role in iron metabolism by producing Tf, the iron carrier protein, and hepcidin, a hormone involved in regulating iron metabolism. As we reported previously, hepcidin is mainly synthesized in hepatocytes, secreted from hepatocytes, and excreted through the kidney. Schematically, extracellular circulating iron in the plasma is present as soluble Tf-bound iron, and when there is excess iron, it binds to serum proteins or citric acid. Human serum albumin is known to be a low-affinity iron-binding protein and it has been proposed as a ligand for the non-transferrin-bound iron (NTBI) pool that exists in the sera of iron-overload patients. Hepatocytes express both TfR1 and TfR2. TfR1 expression is controlled by the level of iron within cells, and is regulated by the iron-regulatory proteins (IRP)–iron-responsive elements (IRE) regulatory system (Cairo & Recalcati, 2007). Fe^{2+} -Tf binds with TfR1 on the cell membrane surface to form a complex that, as mentioned above, is then transported into hepatocytes by endocytosis. The released ferrous iron is transported from the interior of the endosome to the cytosol by divalent metal transporter 1 (DMT1).

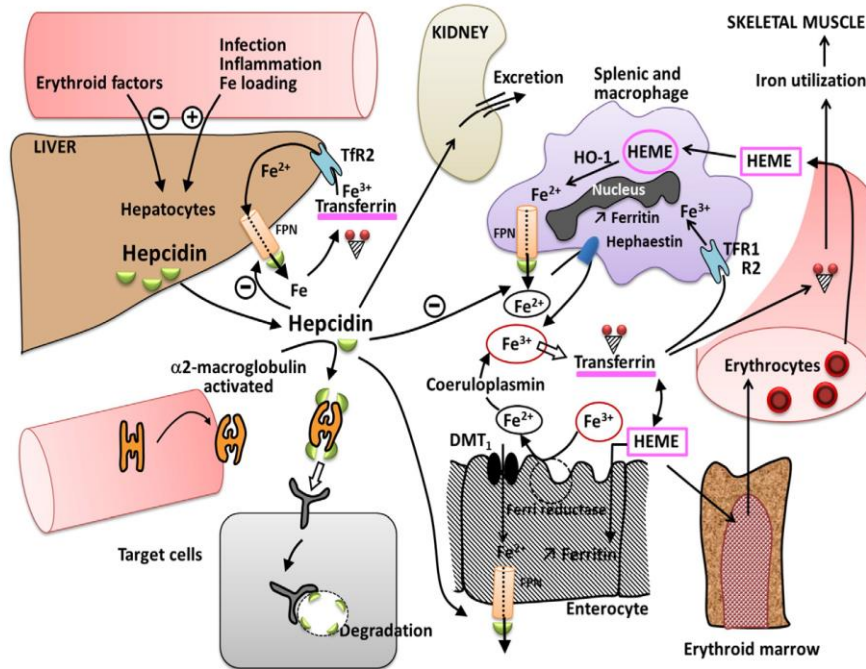


Fig. 1. Regulation of systemic iron metabolism and hepcidin expression. Enterocytes incorporate iron via divalent metal transporter 1 (DMT1) located on the apical membrane upon reduction of Fe³⁺ to Fe²⁺ by ferri-reductase. Hepatic hepcidin regulates the efflux of iron from the cells by regulating the activity of ferroportin (FPN). The synthesis and secretion of hepcidin by hepatocytes are influenced by iron levels in relationship with infection and inflammation. Hepcidin preferentially binds to the blood glycoprotein, activated α 2-macroglobulin (α 2M). Macrophages and spleen recognize damaged or senescent erythrocytes, phagocytize them and digest them to extract heme and iron. Heme is degraded through the action of haem oxygenase-1 (HO-1) to release iron. Macrophages export Fe²⁺ via FPN, in a process coupled by re-oxidation of Fe²⁺ to Fe³⁺. Hephaestin assists by converting Fe²⁺ to Fe³⁺, which is then bound by transferrin (TF). In the circulation, iron is bound to TF. TF possesses receptors (TFR) that mediate iron metabolism.

A growing body of evidence suggests that dysregulation of iron metabolism contributes to age-related pathologies. Hepatocyte senescence correlated positively with hepatic iron concentrations, serum ferritin and oxidative stress. The mechanisms that contribute to iron retention with ageing are unclear, but might involve enhanced iron uptake, or reduced iron export in these tissues, potentially resulting from the dysregulation of iron transport proteins (Wood et al., 2014). Iron retention generates oxyradicals and contributes to the peroxidation of lipids and proteins in membranes, thus leading to cellular toxicity. This process is thought to contribute to hepatocellular necrosis and apoptosis and the development of hepatic fibrosis in pathologies associated with iron retention (Ramm & Ruddell, 2010). The hypothesis according to which iron overload could be harmful has been extensively and controversially discussed in relationship with oxidative stress. In patients, the correlation between haemochromatosis and an established marker of oxidative stress, the urinary excretion of 8-iso-prostaglandin F₂ α has been demonstrated (Kom et al., 2006). A recent study in rats investigated whether or not iron supplement facilitated the onset of induced type-2 diabetes by the overproduction of reactive oxygen species (ROS) and reactive nitrogen species (RNS) and exacerbated oxidative/nitrosative stress in association with hepatic dysfunction. Both diabetes and elevated amounts of liver iron caused hepatic disease, resulting in increased liver injury including fibrosis, inflammation and hepatocellular necrosis (Li et al., 2012). These results were similar to the

conclusions of studies in animal models of haemochromatosis (Klopffleisch & Olias, 2012). It is known that mutations in HFE or TFR2 cause hereditary haemochromatosis by impeding production of the liver's iron-regulatory hormone, hepcidin. The effects of disrupting HFE or TFR2, either alone or together, on liver iron loading and injury in mouse models of hereditary haemochromatosis were investigated. Liver F2-isoprostane levels were higher, and copper/zinc and manganese superoxide dismutases (SODs) activities lower in mutant animals than in wild-type mice (Delima et al., 2012). In conclusion, there is now strong evidence that iron overload plays an important role in the initiation and progression of hepatic injury through its ability to catalyze the formation of ROS thus inducing oxidative stress that is not compensated for by endogenous antioxidant mechanisms.

5. Levels, structures, and kinetics of hepcidin

5.1. Levels of hepcidin

Hepcidin was discovered by three laboratories working independently (Krause et al., 2000; Park et al., 2001; Pigeon et al., 2001). The laboratory of Tomas Ganz invented the name hepcidin, because the gene is highly expressed in the liver (hep-) and was found to possess some microbicidal activity (-cidin). The bacteriostatic effects of iron-binding proteins had already been recognized in the 1940s. It was

noted that specific iron-binding proteins in egg white (ovotransferrin) and blood (transferrin) inhibited the growth of certain bacteria—an effect easily reversed by adding iron. There are considerable differences in hepcidin levels depending on the method used to make the measurement. Absolute hepcidin concentrations differed widely between the assays (Laarakkers et al., 2013).

To date, concentrations of the biomarker hepcidin have only been assessed in the sera of relatively small series of healthy volunteers and patients. In a large, well-phenotyped sample of the general population ($n = 2998$), a recent study assessed age- and sex-stratified reference ranges of serum hepcidin concentrations. Variations in hepcidin concentrations over age differed between men and women. Men showed a stable hepcidin concentration, although a non-significant trend towards an age-related increase in serum hepcidin was previously reported in a study of 65 men. In women, serum hepcidin concentration was substantially higher for postmenopausal than for premenopausal women. The median serum hepcidin concentration in men was 7.8 nM, while that in women was 4.1 nM for women younger than 55 years and 8.5 nM for women aged 55 years and older (Galesloot et al., 2011). There is currently no evidence to support either a primary or secondary circadian variation in hepcidin. However, circadian variations in hepcidin do exist and could be secondary to variations in iron intake during the day. Serum ferritin was shown to be the most important correlate of serum hepcidin concentration (Ashby et al., 2009). In the Nijmegen Biomedical Study, hepcidin and the hepcidin/ferritin ratio, which reflects hepcidin expression relative to iron stores, were significantly associated with the presence of atherosclerotic plaque in postmenopausal women (Galesloot et al., 2014). It has been hypothesized that high levels of hepcidin may increase cardiovascular risk by slowing or preventing the mobilization of iron from macrophages. (See later paragraph: Hepcidin: endogenous cytoprotective agent in cardiovascular pathophysiology and possible therapeutic targets).

5.2. Structure of hepcidin (Fig. 2)

Hepcidin is synthesized in the liver as an 84-amino-acid (AA) preprohormone, and is targeted to the secretion pathway by a 24-AA N-terminal targeting sequence. The resulting 60-AA pro-hepcidin is processed further into a mature C-terminal 25-AA active peptide (~2.7 kDa) that is secreted into the circulation. The human 25-amino-acid hepcidin peptide contains four disulphide bonds, and urinary hepcidin is rich in β -sheet. Hepcidin forms a simple hairpin stabilized

by four disulphide bonds (Ganz, 2005). Under physiological conditions, N-terminal truncated hepcidin-20, -22 and -24 peptides have been observed in the urine, but are absent from or exist at low concentrations in plasma. These smaller hepcidin isoforms mostly occur in plasma in diseases that are associated with significantly increased hepcidin concentrations, such as sepsis and kidney failure. Hepcidin concentrations were reported to be increased in patients with chronic kidney disease. This could be caused by inflammation, which frequently accompanies renal failure. However, even patients without significant inflammation have elevated hepcidin levels that progressively increase with the severity of the kidney disorder. Because hepcidin is cleared, at least partially, by filtration in the kidney, decreased kidney function probably contributes to this phenomenon. This hypothesis is supported by a number of studies that reported an inverse correlation between the glomerular filtration rate and the serum concentration of hepcidin (Peters et al., 2010). Hepcidin-25 has both antimicrobial and iron-regulatory activities, whereas hepcidin-22 and hepcidin-20 have only antimicrobial activity. Much is still unknown about the origin of the smaller isoforms. During prolonged storage of plasma at room temperature, it has been observed that a decrease in plasma hepcidin-25 was paralleled by an increase in the levels of the hepcidin-24, -22 and -20 isoforms. This means that all of the determinants for the conversion of hepcidin-25 to smaller inactive isoforms are present in the circulation, which may contribute to the functional suppression of hepcidin-25, which is significantly higher in patients with renal impairment (Laarakkers et al., 2013). We found that hepcidin was expressed predominantly in the hepatocytes around the portal veins in mouse liver. Interestingly, ferroportin staining was also found to be much more prominent in the periportal areas.

5.3. Hepcidin kinetics

Binding to carrier proteins may prevent circulating hepcidin from being freely filtered (Peters et al., 2010). Hepcidin preferentially binds to the blood glycoprotein, α_2 -macroglobulin (α_2 M) and to methylamine-activated α_2 M (α_2 M-MA). α_2 M, which is composed of subunits with tetrameric structures in vertebrates, protects the body against various infections, and hence, can be used as a biomarker for the diagnosis and prognosis of a number of diseases (Rehman et al., 2013). Hepcidin binds to the methylamine-activated form of α_2 M via four high-affinity binding sites. In addition to hepcidin binding, both α_2 Ms bind many hormones and cytokines such as IL-6. Hepcidin complexed with native and activated α_2 M significantly decreased FPN

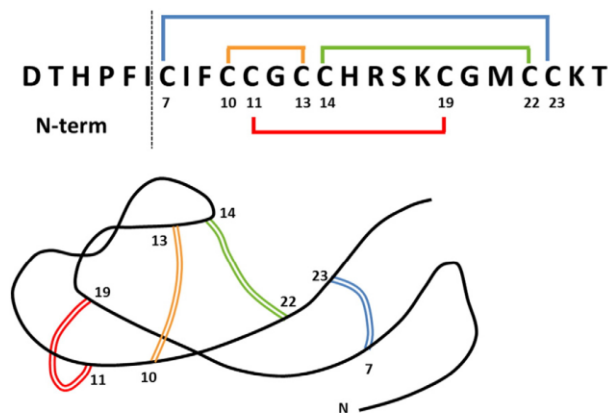


Fig. 2. Hepcidin: the amino-acid sequence and structure. Hepcidin is synthesized as an 84-amino-acid (AA) preproprotein. A furin-like proprotein convertase is responsible for cleavage of pro-hepcidin to hepcidin. The human 25-amino-acid hepcidin peptide contains four disulphide bonds. N-terminal domain interacts with ferroportin.

expression. Hecpudin bound to activated $\alpha_2\text{M}$ binds to the $\alpha_2\text{M}$ receptor: low density lipoprotein receptor-related protein-1 (Lrp1) on target cells. Activated $\alpha_2\text{M}$ is internalized by endocytosis via these receptors (Huang et al., 2013).

Hecpudin clearance is assumed to occur via cellular co-degradation with FPN at its sites of action, and via excretion by the kidneys. Because of its low molecular weight and small radius, unbound hecpudin probably passes freely into the glomerular filtrate. Possible local tubular production of hecpudin as well as possible defective tubular reabsorption of hecpudin must also be taken into account in the interpretation of urine levels as a mirror of serum hecpudin concentrations (Kulaksiz et al., 2005). Recently, hecpudin-25 has been shown to have a direct correlation with creatinine and an inverse correlation with estimated glomerular filtration rate, suggesting that hecpudin-25 levels increase as renal function deteriorates, possibly due to decreased renal clearance of hecpudin-25 (Troutt et al., 2013).

6. Sources of extrahepatic hecpudin

Apart from hepatocytes, which are the main source of circulating hecpudin, other cell types such as macrophages, adipocytes, and heart and stomach cells express hecpudin mRNA, but at a lower level. While extrahepatic hecpudin expression is becoming established, the physiological role of hecpudin in extrahepatic tissues is only partially understood. Extrahepatic hecpudin production may play a role in the local regulation of iron fluxes.

6.1. Macrophages

Macrophages facilitate the rapid throughput of iron and strongly express FPN. The transport of iron across vacuolar membranes into the cytoplasm of macrophages involves DMT1, which is specific for ferrous iron. The necessary iron reduction is catalyzed by an erythroid endosomal ferric reductase, which is important for iron utilization in erythrocytes.

A sequence of events takes place in macrophage lysosomes when they phagocytose senescent erythrocytes. The hydrolytic environment of the phagolysosome digests the erythrocyte and its haemoglobin, releasing haem, which is then degraded by inducible haem oxygenase-1 (HO-1). Iron is released for cytoplasmic storage or export to blood plasma; these processes may be particularly influenced by hecpudin. HO-1 acts within the phagolysosomes, and the released iron is transported across the phagolysosomal membrane. Macrophages are important producers of hecpudin, and within macrophages, hecpudin localizes in mycobacteria-containing phagosomes where it exerts its antimycobacterial activity (Layoun & Santos, 2012). In macrophages, the regulation of lipopolysaccharide (LPS)-inducible cytokines, which include several endogenous compounds such as TNF α , interleukin (IL) families and receptors, especially Toll-like receptors (TLRs) and IL-1R takes place. LPS-induced hecpudin expression *in vivo* is dependent on TLR4, indicating that TLR signalling may be important for the activation of hecpudin production in macrophages (Fig. 1) (Constante et al., 2006). In contrast to hecpudin expression in hepatocytes, that in macrophages is positively regulated by gram-positive and gram-negative bacteria wall constituents through TLR2 and TLR4 (Layoun & Santos, 2012). Hecpudin expression by macrophages increases following bacterial infection, and hecpudin decreases iron release from macrophages in an autocrine and/or paracrine manner. Though circulating hecpudin seems to be a relatively ineffective antimicrobial peptide, it was recently proposed that hecpudin produced endogenously by macrophages and localized in the phagosome could play a role in host defences against tuberculosis by causing structural damage to the mycobacteria (Sow et al., 2011).

In monocytes, autocrine production of hecpudin has been shown to down-regulate FPN expression, thereby contributing to iron sequestration within these cells (Theurl et al., 2008). The hecpudin-mediated elevation of intracellular iron plays an important role in the cytokine

response of macrophages and hence subsequent immune responses (Layoun & Santos, 2012). It has recently been proposed that High Mobility Group Box-1 (HMGB1), which is released from irradiated tumour cells, acts as an endogenous TLR4 ligand (Apetoh et al., 2007). HMGB1 is a nuclear protein that can be released into the extracellular milieu from immune and non-immune cells in response to various stimuli. Extracellular HMGB1 contributes to the pathogenesis of numerous chronic inflammatory and autoimmune diseases, including sepsis, rheumatoid arthritis, atherosclerosis, chronic kidney disease and cancer. It has been suggested that there is some interplay between HO-1 and HMGB1 (Gong et al., 2008).

In atherosclerotic plaque, iron preferentially accumulates in macrophages where it can exert pro-oxidant activities (Yuan & Li, 2003). The physiology of hecpudin suggests an additional mechanism by which iron depletion could protect against the progression of atherosclerotic lesions. Without hecpudin, macrophages retain less iron. Hecpudin may promote plaque destabilization by preventing iron mobilization from macrophages within atherosclerotic lesions. This process can be altered and the absence of this mobilization may result in increased cellular iron loads, thus inducing lipid peroxidation, which is associated with progression to foam cells (Sullivan, 2007).

Interestingly, it was recently demonstrated that after exposure to iron, differentiated macrophages (M2) completely changed their phenotype and acquired a phenotype oriented to iron release. The iron handling ability of M2 macrophages suggests they play a modulatory role in atherosclerosis. Hecpudin decreased iron loading in M2 macrophages, whereas FPN expression increased. Expression of the HO-1 gene and NRF2, which are markers of oxidative stress, also increased markedly in M2 macrophages. Finally, these M2 macrophages are highly specialized in iron recycling associated with increasing FPN expression and a decrease in hecpudin mRNA levels (Bories et al., 2013).

6.2. Adipocytes

Recent data suggest that adipocytes are capable of producing hecpudin, and that this process is probably triggered by inflammatory stimuli. Hecpudin mRNA and hecpudin were present in adipose tissue, and mRNA levels in adipose tissue correlated with levels of IL-6 and C-reactive protein (CRP) (Bekri et al., 2006). Adipose tissue expressed hecpudin at both the mRNA and protein level and this expression was enhanced in obese patients (Vuppalanchi et al., 2014). A lot of results point out that hecpudin may play an important role in the hypoferrremia of inflammation in obese patients. There is a tight relationship between iron deficiency and obesity. Obesity seems to be associated with lower iron levels and higher iron stores, reflecting lower iron bioavailability, together with higher hecpudin production and with higher levels of obesity-related inflammation markers (Coimbra et al., 2013). In this field, it has been reported that activin B was a new adipokine having a role in energy balance and insulin insensitivity associated with obesity (Dani, 2013). Interestingly, hecpudin synthesis was induced by inflammatory signals including activin B and SMAD responded to activin B (Besson-Fournier et al., 2012; Akel et al., 2013).

Interactions between cells in the blood vessel wall and regional lipids are vital for normal vascular function, and they are disturbed in cardiovascular diseases. Perivascular adipocytes are present at the adventitial border of blood vessels. The secretion of mediators that regulate inflammation is a characteristic feature of adipocytes. These mediators can be divided into two categories: adipokines and cytokines. Adipose tissue is known to secrete IL-6, leptin and adiponectin (Rajsheker et al., 2010). As we reported, hecpudin is rapidly induced by cytokines, including IL-6. In human volunteers, recombinant IL-6 infusion resulted in a 7.5-fold increase in urine hecpudin levels, and serum iron decreased by 34% within 2 h. IL-6 neutralizing antibodies were used to show that there was an IL-6-dependent inflammation-mediated and regulatory-hecpudin pathway (Nemeth et al., 2004a). It has been suggested that adipocytes are key regulators of metabolism in all tissues,

given their integrated sensing of nutritional stores and iron availability. Evidence now exists that adipocyte iron levels regulate adiponectin transcription and serum protein levels (Gabrielsen et al., 2012). High levels of leptin are found in obesity and also appear to contribute to the iron status observed in obesity. Leptin is capable of inducing hepatic hepcidin expression via the Janus kinase 2 (Jak2)/STAT3 signalling pathway, which is similar to IL-6-mediated hepcidin expression. STAT3 belongs to the signal transducer and activator of transcription (STAT) family of signal responsive transcription factors. In non-stimulated cells, STAT3 is kept in an inactive cytoplasmic form. The JAK2/STAT3 pathway is a stress-response mechanism that transduces signals from the cell surface to the nucleus, thereby modulating gene expression (Chung et al., 2007; Meli et al., 2013). Certain studies have reported that circulating hepcidin and leptin concentrations in obese patients are higher than those in normal-weight subjects. These observations support the idea that the poor iron status frequently observed in obese patients may be associated with the hepcidin-mediated inhibition of dietary iron absorption (Amato et al., 2010). As we previously reported in the underlying mechanism of iron-regulated hepcidin expression, several proteins that participate in this process have been identified. Among these, haemojuvelin (HJV) plays a particularly important role. A soluble form of haemojuvelin (s-HJV) exists in the blood. Recently, it has been reported that HJV was also expressed in adipocytes at mRNA and protein levels. Moreover, its mRNA expression was greatly increased in adipose tissue from obese individuals and correlated positively with hepcidin expression levels (Luciani et al., 2011).

6.3. Heart

It has been reported that increased levels of iron and iron-mediated injury play an important role in the development of a number of heart disorders, including iron-overload cardiomyopathy, acute myocardial infarction and coronary heart diseases (Tuomainen et al., 1998; Rochette et al., 2011). Heart cells have the ability to accumulate transferrin-bound iron via the Tf receptor and non-transferrin-bound-iron probably via the L-type Ca^{2+} channel. FPN-mediated iron export is dependent on the ferroxidase activity of the multicopper oxidases, ceruloplasmin and hephaestin (Qian et al., 2007). It has also been demonstrated that hepcidin was expressed in the heart; this implies that this protein might play a role in iron homeostasis in the heart. In vitro, it has been demonstrated that hepcidin was able to bind with FPN, internalize and degrade FPN, and thus decrease iron export from heart cells (Merle et al., 2007; Ge et al., 2009).

In our laboratory, we found that hepcidin was present in the myocardium. The protein was diffusely expressed in the myocardium (Fig. 3). Recently, cardiac expression of hepcidin in different conditions: rat experimental autoimmune myocarditis (EAM), rat acute myocardial infarction (AMI) and human myocarditis have been investigated. Hepcidin mRNA was moderately expressed in the heart of normal rats, but the expression of hepcidin mRNA in the heart of EAM rats was markedly greater than that in controls. In humans, the level of hepcidin/cytochrome oxidase mRNA in hearts with pathologically diagnosed myocarditis was significantly higher than that in hearts without

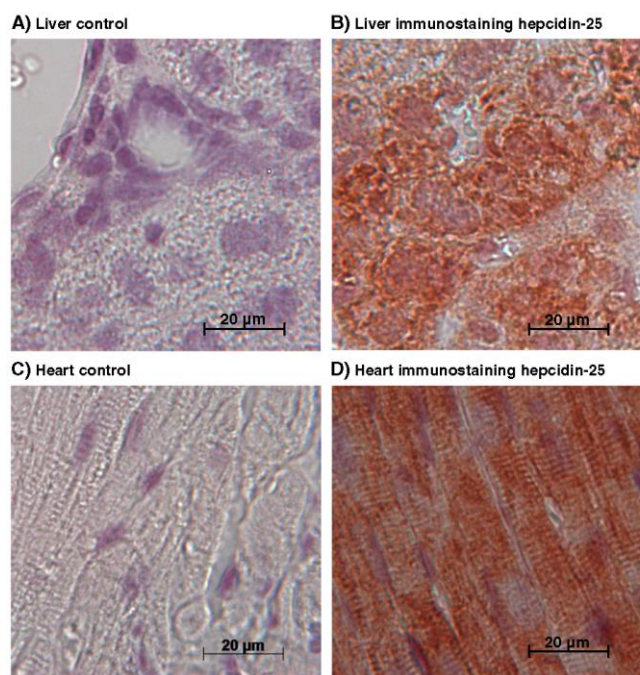


Fig. 3. Histology: Hepcidin expression in the liver and heart. Representative images of hepcidin-25 immunostained liver and heart sections. "After fixation in formalin and dehydration in alcohol, the samples were embedded in paraffin. Slices of 5 µm were cut and immunostained for hepcidin. Briefly, after antigen retrieval (citrate buffer pH6), slices were incubated with H_2O_2 3% in TBST (2×8 min) and saturated (Impress Kit serum plus BSA 3% in TBST) for 20 min. They were incubated with anti-hepcidin (1/50, Abcam—ab30760) overnight at 4 °C. After 3 rinses in TBST, the slices were incubated with Impress Kit Solution (Vector Lab—MP7401) for 30 min. After the revelation step with Nova Red kit (Vector Lab—SK4800), they were counterstained in haematoxylin."

myocarditis. Finally, hepcidin expression is more strongly influenced by local environmental conditions than by systemic conditions; hepcidin mRNA levels correlated with those of IL-6 mRNA (Isoda et al., 2010). In hypoxic rat hearts, expression levels of hepcidin were highest at the myocardial intercalated disc area (Merle et al., 2007). Given this localization, it is logical to conclude that the presence of hepcidin in the intercalated disc area may be an important component in a local response to an inflammatory process.

Another condition that induces hepcidin expression is inflammation associated with cardiac diseases such as heart failure. Increases in pro-inflammatory cytokine levels and anaemia have been reported in patients with heart failure. It is possible that the increased level of inflammatory cytokines in the heart may induce hepcidin gene expression in cardiomyocytes (Nemeth et al., 2003). A large number of transgenic over-expression studies that investigated a wide array of intracellular signalling pathways in the hypertrophic response have been published. Using transgenic models, it appears that hepcidin in the heart plays a role in the regulation of iron homeostasis in an autocrine and paracrine fashion (Zhang et al., 2012).

6.4. Stomach

Given the established role of the stomach and gut in iron metabolism, it is tempting to speculate that the hepcidin system is implicated in the regulatory and antimicrobial functions of iron. A recent study showed that hepcidin was expressed in gastric parietal cells (Schwarz et al., 2012). In mice and rats, quantitative RT-PCR analyses revealed abundant hepcidin expression in the fundus/corpus part of the glandular stomach, and less ample expression in the antrum. The influence of hepcidin on gastric acid secretion and the regulation of gastric acid production may also contribute to local iron homeostasis. Hepcidin is also present in the bile (Strnad et al., 2011). Hepcidin was increased after treatment with proton pump inhibitors and reduced after the injection of histamine; these results suggest that hepcidin signalling serves as a mechanism to keep gastric acid secretion steady under normal and pathological conditions such as inflammation. Iron absorption is diminished during inflammation, and the mechanisms operating in the intestine to decrease iron absorption during inflammation have been poorly studied at the cellular level. Treatment with TNF alpha decreased intestinal iron absorption, but TNF appears to mediate hypoferrremia independently of hepcidin (Schubert et al., 2012). One study investigated

whether hepcidin was implicated in colon cancer-associated anaemia and whether it might play a role in colorectal carcinogenesis. Hepcidin is expressed by colorectal cancer tissue and thus may be a novel oncogenic signalling mechanism (Ward et al., 2008).

7. Importance of the hepcidin-ferroportin interaction (Fig. 4)

The interaction of hepcidin with FPN provides a mechanism for coordinating the entry of iron into the plasma with iron utilization and storage. Recent advances in understanding the molecular mechanisms of hepcidin regulation in particular in relationship with FPN and its targets came from studying patients with a genetic iron-overloaded disorder such as haemochromatosis. These patients present mutations in genes that encode different proteins and they have low hepcidin levels relative to iron stores (Gao et al., 2009).

The main target of hepcidin is FPN, the transmembrane protein responsible for iron export from cells to plasma (Nemeth et al., 2004b). FPN protein is encoded by the SLC40A1 gene located on chromosome 2 (2q32). FPN gene mutations have been identified in different ethnic populations and some of the mutations are associated with autosomal dominant iron-overload disorder. To date, forty-five mutations in the SLC40A1 gene have been described in people of different ethnicity, and this number is set to increase as more mutations are discovered. In the majority of cases, FPN polymorphism is a result of a missense mutation (Kasvosve, 2013). Mutations in the FPN gene lead to autosomal dominant iron-overload diseases (Detivaud et al., 2013). Hepcidin binds to a highly conserved domain on an extracellular loop of FPN. The extracellular loop of FPN that interacts with hepcidin was identified from the observation that the Cys326 mutation in this loop resulted in FPN being resistant to hepcidin binding. It has been proposed that the free thiol of the Cys326 residue forms a disulphide bond with one of the cysteine residues in hepcidin (Clark et al., 2011). The concentration of FPN on the surface of cells depends on the rate of synthesis, the hepcidin/FPN interaction, the rate of internalization and the rate of cellular degradation. Once internalized, FPN is dephosphorylated and subsequently ubiquitinated. The inability to ubiquitinate FPN does not prevent hepcidin-induced internalization, but it inhibits the degradation of FPN. Ubiquitinated FPN is trafficked through the multivesicular body pathway en route to degradation in the endosome (De Domenico et al., 2007).

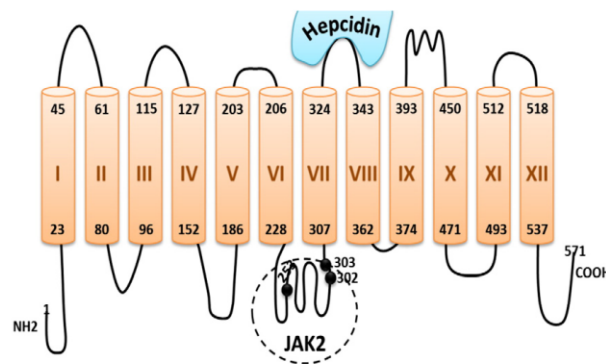


Fig. 4. Human ferroportin and functional domains (12 transmembrane regions that were 21–23 amino acids long). Hepcidin binds to a domain on an extracellular loop of ferroportin. Hepcidin exerts its influence on iron metabolism via down-regulation of FPN-mediated iron export. Janus kinase 2 (JAK2) binds to a cytoplasmic domain of ferroportin.

It has been demonstrated that the kinase responsible for the phosphorylation of FPN is JAK2. Binding of JAK2 to FPN is highly cooperative and requires hepcidin to bind to both monomers of the FPN dimer and both monomers to be capable of activating JAK2 (De Domenico et al., 2009). Studies have suggested that a thiol-disulphide exchange may occur during hepcidin binding to FPN (Preza et al., 2011). Once bound, JAK2 is autophosphorylated and then phosphorylates FPN, leading to FPN internalization (De Domenico et al., 2009).

In summary, excess iron induces an increase in hepcidin concentration, resulting in the internalization and degradation of FPN protein. Intracellular iron is then sequestered by binding to ferritin within cells, limiting the increase in plasma iron concentration and Tf saturation. Coupling the internalization of FPN to hepcidin levels could generate a homeostatic loop that regulates iron plasma levels and the tissue distribution of iron.

8. Hepcidin: master regulator of iron metabolism

When iron stores are adequate or high, the liver produces hepcidin, which circulates to the small intestine. FPN molecules are expressed on the basolateral membranes of enterocytes, and they transport iron from enterocytes to plasma transferrin. Hepcidin causes FPN to be internalized, thus blocking the pathway for the transfer of iron from enterocytes or macrophages to plasma. Macrophages export Fe^{2+} from their plasma membrane via FPN, in a process coupled with the re-oxidation of Fe^{2+} to Fe^{3+} by ceruloplasmin and followed by the loading of Fe^{3+} onto Tf. Exported iron is scavenged by Tf, which maintains Fe^{3+} in a redox-inert state and delivers it into tissues. The copper-containing ferroxidase, hephaestin, assists by converting Fe^{2+} to Fe^{3+} , which is then bound by Tf.

As we previously reported, iron enters the cell from the bloodstream in a complex with Tf, which binds to receptors TFR1 and TFR2 on the plasma membrane, followed by receptor-mediated endocytosis. As stated above, human TFR1 and TFR2 are very similar, being 45% homologous and 60% similar in their primary structure (Anderson & Vulpe, 2009; Bayeva et al., 2013; Koskenkorva-Frank et al., 2013). Under physiological conditions, plasma Tf is hyposaturated (to ~30%) and displays a very high iron-binding capacity.

Different studies have revealed that expression of the hepcidin–FPN axis was regulated by different factors (exogenous and endogenous). At the molecular level, the pathways involved in this regulation are not well known. The effect of vitamin D on the hepcidin–FPN axis suggests that low vitamin D status may be a contributing factor to the anaemia of chronic kidney disease (CKD), which is characterized by impaired vitamin D status, which itself is closely associated with adverse CKD health outcomes. Recently, a study showed for the first time that vitamin D was a potent regulator of hepcidin in both monocytes and hepatocytes (Bacchetta et al., 2014). In conditions of vitamin D deficiency, elevated synthesis of hepcidin by hepatocytes may increase intracellular and systemic concentrations of hepcidin and thus decrease membrane expression of FPN in these cells. Direct transcriptional suppression of hepcidin gene (HAMP) expression mediated by 1,25-dihydroxyvitamin D binding to the vitamin D receptor causes a decrease in hepcidin mRNA levels.

Studies have reported a decrease in hepcidin expression in response to hypoxia. However, the physiological relevance of hypoxia and its impact on hepcidin regulation are still uncertain and conflicting. In vivo, hypoxia could also suppress hepcidin indirectly through erythropoiesis and enhanced iron use. Hypoxia-inducible factor (HIF) may, however, contribute to hepcidin suppression indirectly via its effects on the breakdown of haemojuvelin (Silvestri et al., 2008). Moreover, HIF1 α and 2 α were shown to increase intestinal iron absorption, iron uptake into erythroid progenitors and haem synthesis, and to suppress hepcidin production thus ensuring an adequate supply of iron to support erythropoiesis (Evstatiev & Gasche, 2012; Palaneeswari et al., 2013). In the HIGHCARE study (HIGH altitude Cardiovascular Research carried out 3400–5400 m above sea level) the modifications induced

by acute and chronic exposure to hypobaric hypoxia on serum iron indices, EPO, IL-6, and hepcidin levels were investigated. Hepcidin levels decreased within 40 h after acute hypoxia exposure at 3400 m and reached the lowest level at 5400 m. This fall was associated with a rapid decrease in serum levels of ferritin. The strong correlation between serum ferritin and hepcidin during the study indicates that iron itself or the kinetics of iron use in response to hypoxia may signal hepcidin down-regulation (Piperno et al., 2011).

In the process of hepcidin regulation, the endoplasmic reticulum may play a role with regard to inflammation. The acute inflammatory response has been linked to endoplasmic reticulum (ER) stress, a state that is associated with disruption of ER homeostasis and the accumulation of unfolded or misfolded proteins in the ER. Overall, it appears that the regulation of hepcidin by ER stress links the cellular response involved in protein quality control to innate immunity and iron homeostasis. Apparently, hepcidin reacts not only to extracellular stimuli, such as iron fluctuations and cytokines, but also to stress signals arising within the cell (Vecchi et al., 2009; Oliveira et al., 2011).

Among the endogenous “mediators”, carbon monoxide (CO) and NO appear to play a role in this regulation. CO suppresses hepcidin expression elicited by IL-6 and endoplasmic reticulum-stress agents by inhibiting signal transducer and activator of transcription (STAT-3) phosphorylation and cyclic AMP response element-binding protein-1 (CREB1) maturation, respectively. The induction of endoplasmic reticulum stress in mice resulted in increased hepatic and serum hepcidin (Shin et al., 2012). The anti-inflammatory action of CO is implicated in these different processes (Rochette et al., 2013).

Concerning NO, it has been hypothesized that iron homeostasis and NO biology are interconnected (see previous paragraph: Role of Ferritin). As we previously mentioned, the regulation of ferritin synthesis by iron is mainly due to post-transcriptional regulation through the binding of IRP1 and IRP2 to IRE and the NO-induced expression of FPN (Straub et al., 2012; Nairz et al., 2013). Several reports indicate a potential synergistic effect between NO and H_2S in controlling various biological responses of vascular function. It is interesting to note that the cytoprotective functions of low concentrations of H_2S are comparable to those of NO. Importantly, NO and hydrogen can react rapidly with metalloproteins such as iron–sulphur clusters (Kolluru et al., 2013; Yin et al., 2013).

9. Regulation of hepcidin expression (Fig. 5)

The expression of hepcidin is dependent on opposing signalling pathways: the combined effects of the various pathways will determine hepcidin levels. The systemic factors that alter hepcidin expression, such as anaemia or inflammation, are well established. However, the mechanism by which hepcidin production is regulated at the molecular level is still unclear.

HFE, the gene mutated in the most common form of hereditary haemochromatosis, plays a role in monitoring body iron status and then directing the adaptive hepcidin response. The HFE protein interacts with TFR1 at a site that overlaps with the binding site for Tf. A close correlation between Tf levels and hepatic hepcidin mRNA expression has been demonstrated following haemolysis (Frazer et al., 2004). Furthermore, when Tf binds to TFR2, the ERK1/ERK2 and p38 MAP-kinase pathways are activated, thus inducing hepcidin expression (Calzolari et al., 2006). As mentioned above, inflammation stimulates hepcidin expression, which in turn causes the hypoferrremia associated with inflammatory episodes. Although several pro-inflammatory cytokines have been shown to increase hepcidin expression, IL-6 has been the best studied. It stimulates hepcidin transcription through STAT3 signalling. Several microbial-derived Toll-like receptor (TLR) ligands can induce hepcidin expression, perhaps via the induction of IL-6 (Darshan & Anderson, 2009). The most recently described factor in the regulatory pathway in hepcidin expression is the BMP (bone morphogenetic protein)/SMAD (contraction of Sma and Mad) pathway: BMP/

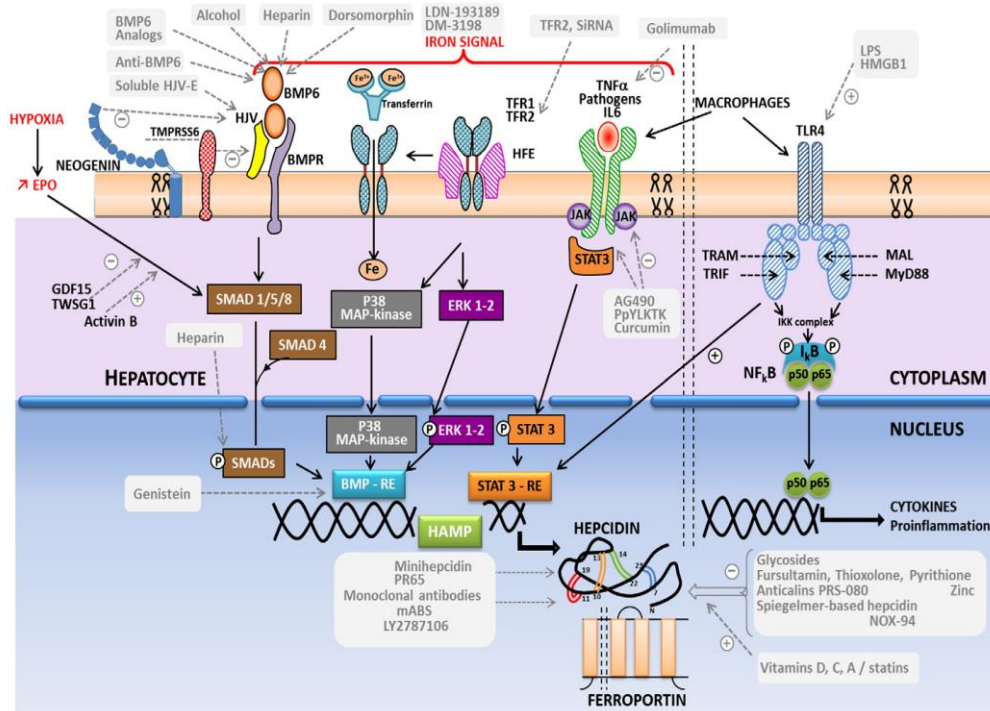


Fig. 5. Expression of hepcidin in hepatocyte and macrophages in response to iron signal and targeting of the hepcidin–ferroportin axis for human therapy. Hepcidin gene expression is up-regulated by inflammation and iron through the JAK/STAT and BMP/SMAD pathways respectively. The BMP-6 signal acts through its receptor (BMPR), and is modulated by haemojuvelin (HJV). TSPRSS6 cleaves membrane-bound HJV. BMPs can also signal through SMAD-independent pathways, notably via MAP kinases. Dorsomorphin inhibits BMP signalling through the SMAD pathway. SMAD complexes bind to BMP-responsive elements (BMP-Res). TNF, pathogens and IL-6 stimulate hepcidin synthesis via STAT-3 activation. The p38, MAP-kinase and ERK 1–2 pathways are activated in response of iron signals. Ferric transferrin (Tf) binds to Tf-Receptor 1 (TfR-1) on the cell surface and the complex undergoes endocytosis. HFE is a protein that competes with Tf for binding to TfR1. TLR signalling requires interactions of the receptor domains of the receptor and adaptor proteins. Hepcidin expression in macrophages is regulated mainly through TLR4 receptors associated with adaptor proteins (TRAM, TRIF, MAL, MyD88). Hepcidin mRNA expression in macrophages induced by LPS or HMGB1 depends on NF-κB. For simplicity, not all factors involved with expression are described in this figure. Pharmacological options (shaded parts). BMP-RE: bone morphogenetic protein-responsive element; hepcidin gene (*HAMP*; 19q13) HAMP: The HAMP (present in histidine kinases, adenylate cyclases, methyl accepting proteins and phosphatases).

SMAD. It has also been demonstrated that dorsomorphin, a selective inhibitor of BMP-responsive SMAD phosphorylation, blocked the IL-6 mediated induction of hepcidin (Yu et al., 2008). It appears that the coordinated regulation of hepcidin expression by BMP and IL-6 may involve crosstalk at the level of signal transduction. These effects are influenced by the cellular microenvironment. The human genome encodes 20 B.P. (Miyazono et al., 2010). BMP6 is the specific endogenous regulator of hepcidin (Camaschella, 2009). Mutations in the genes encoding the ligand BMP6, the BMP co-receptor HJV, and the intracellular signalling molecule SMAD4 are associated with suppressing hepcidin expression. These actions are associated with tissue iron overload.

It is now recognized that the BMP6–HJV–SMAD signalling pathway plays a major role in hepcidin regulation and iron homeostasis (Babitt & Lin, 2010). Stimulation by BMP6 and/or iron induces an increase in BMP6–HJV–SMAD pathway activity, possibly through a mechanism involving HFE and TfR2, leading to binding of SMAD complexes to BMP-responsive elements (BMP-REs) on the hepcidin promoter and the up-regulation of hepcidin transcription. The BMPs act by binding to complexes of two type I and two type II receptors (BMPR-I and BMPR-II) and modulating the expression of target genes through

different signal transduction pathways. Signalling through the SMAD proteins is now characterized. The constitutively active kinase domains of type II receptors phosphorylate type I receptors, and this in turn activates the SMAD signalling pathway through phosphorylation of receptor SMADs (SMAD1, SMAD5 and SMAD8). These associate with co-SMADs (SMAD4) to form a heteromeric complex that translocates to the nucleus and stimulates the expression of a wide range of target genes, including the gene encoding hepcidin (Anderson & Darshan, 2008). BMPs can also signal through SMAD-independent pathways, notably via MAP kinases (Nohe et al., 2004).

There are important interactions between the different pathways, and the involvement of other members of the regulatory network, such as growth differentiation factor-15 (GDF-15), has also been reported. GDF15 expression is associated with cellular stress or apoptosis in several tissues. The unprocessed translated form of GDF-15 (pre-pro-GDF-15) is 308 AA long and includes the signal sequence (29 AA), the propeptide (167 AA) and a mature protein (112 AA), which contains a cysteine that binds for the transforming growth factor-β superfamily (Tanno et al., 2010). GDF-15 has been found to be expressed by mature erythroblasts, which occur in diseases due to increasingly ineffective

erythropoiesis such as thalassemia. It has been demonstrated that GDF-15 protein suppresses hepcidin secretion (Tanno et al., 2007, 2010). Erythroblast expression of a second molecule named twisted gastrulation: Twisted Gastrulation Gene 1 (TWSG1) was explored as a potential erythroid regulator of hepcidin. TWSG1 interferes with BMP-mediated hepcidin expression and may act with GDF15 to deregulate iron homeostasis in thalassemia. SMAD signalling pathways are implicated in the cellular actions of GDF-15 and TWSG1 (Tanno et al., 2009).

Recently, several studies have demonstrated that oestrogens regulate hepcidin expression via G-coupled protein 30 (GPR30)–BMP6-dependent signalling, indicating that oestrogen decreases iron absorption in the intestine. GPR30 has been localized to both the cell plasma membrane and the endoplasmic reticulum and has been reported to mediate the action of oestrogen as a steroid receptor. New data suggest that oestrogen directly participates in the regulation of hepatic hepcidin expression (Ikeda et al., 2012).

As we previously reported, hepcidin expression in macrophages is regulated mainly through TLR2 and TLR4 receptors. The autocrine regulation of iron accumulation in macrophages by hepcidin may affect the levels of pro-inflammatory cytokine production. TLR2- and TLR4-mediated hepcidin up-regulation was completely abrogated in myeloid differentiation factor 88 (MyD88)–/– macrophages, suggesting that MyD88 signalling is required for hepcidin induction through TLRs (Layoun & Santos, 2012). TLR signalling requires interactions of the Toll/IL-1 receptor (TIR) domains of the receptor and adaptor proteins. The interaction surfaces in the TLR4 TIR domain for the TLR4–TLR4, TLR4–MyD88 adapter-like (MAL), and TLR4–TRIF-related adapter molecule (TRAM) interaction have been identified (Bovijn et al., 2012).

All of the clinical observations point to iron-responsive regulatory pathways and suggest that oxidative stress could be involved in IL-6 signalling for hepcidin production. During inflammation, cells are exposed to persistently elevated concentrations of superoxide and H_2O_2 . This condition requires the tight regulation of iron homeostasis to prevent tissue damage via Fenton reactions. In neutrophils and macrophages, the membrane-associated NADPH oxidase (NOX2) generates superoxide that is converted to H_2O_2 by SODs (Brechard & Tschirhart, 2008). Recently, it has been shown that very low concentrations of H_2O_2 were sufficient for the potent up-regulation of hepcidin in hepatoma cells and primary hepatocytes, as H_2O_2 acts synergistically with other inducers of hepcidin, such as IL-6. The effect of H_2O_2 on hepcidin is mainly mediated via STAT3, which is the classical inflammatory pathway for the regulation of hepcidin (Millonig et al., 2012).

10. Pharmacology: targeting the hepcidin–ferroportin axis (Table 1)

The hepcidin–FPN axis is the principal regulator of extracellular iron homeostasis in health and disease. Its manipulation via agonistic and antagonistic pathways is an attractive and novel therapeutic strategy. Hepcidin agonists include compounds that mimic the activity of hepcidin and agents that increase the production of hepcidin by targeting hepcidin-regulatory molecules. Advances are being made in this area through the development of small molecule modulators of hepcidin regulation pathways, neutralizing antibodies to hepcidin, and supra-active mini-hepcidins. Antagonists may prevent hepcidin-mediated FPN internalization by at least three distinct mechanisms: (1) preventing the interaction between hepcidin and FPN, (2) inhibiting hepcidin-induced ubiquitination of FPN, and (3) inhibiting the endocytosis pathway for FPN internalization.

10.1. Direct and indirect hepcidin agonists

10.1.1. Mini-hepcidin: hepcidin analogues (7–9 amino-acid peptides)

The use of natural hepcidin as a potential replacement therapy in hepcidin-deficient conditions has major limitations. The half-life of natural hepcidin is very short due to its rapid renal excretion. Oral

absorption of hepcidin would be low due to its large size (~2.7 kDa). A series of 7- to 9- amino-acid peptides, “mini-hepcidins,” that mimic the activity of hepcidin has been developed. The rationale for their design was facilitated by the identification of the region on hepcidin and FPN molecules that is critical for their binding. Mutagenesis studies and biomolecular modelling indicated that the first 9 amino acids of the hepcidin N-terminus were important for hepcidin activity. Synthetic N-terminal peptides were further engineered to increase their bioavailability (Preza et al., 2011). Unnatural amino acids were introduced to increase resistance to proteolysis, and fatty acids were conjugated to prolong the half-life in the circulation and potentially increase oral absorption. One mini-hepcidin (PR65) has been tested. From the N to the C terminus, the primary sequence of PR65 was (all L-amino acids): iminodiacetic acid, threonine, histidine, diphenylalanine, β -homo proline, arginine, cysteine, arginine, and β -homo phenylalanine. The C-terminal carboxamide was derivatized with polyethylene glycol (PEG) linker and palmitic acid groups (Preza et al., 2011; Ramos et al., 2012). Minihepcidins could be useful for the prevention of iron overload, or may be used in combination with phlebotomy or chelation for the treatment of existing iron overload (Fung & Nemeth, 2013). Recently, a number of cyclic hepcidin analogues have been designed and synthesized with the aim to produce a cyclized hepcidin that maintains activity relative to native hepcidin. Cyclization of hepcidin results in highly stable peptides with a hepcidin-like fold. However, they do not induce FPN internalization in vitro (Clark et al., 2013).

10.1.2. TMPRSS6 a membrane serine protease

TMPRSS6 also called matriptase-2 and produced by the liver is a type II plasma membrane serine protease (TTSP). The 811-AA human protein is synthesized as an inactive zymogen and autoactivated by proteolytic cleavage. TMPRSS6 mRNA expression has been demonstrated to be induced by erythropoietin and hypoxia and by acute iron deprivation at the protein level. Haemojuvelin (HJV) was the first biologically relevant exogenous substrate for matriptase-2 to be identified (Lee, 2009). TMPRSS6 is mutated in iron-refractory iron-deficiency anaemia. It inhibits hepcidin expression by depressing BMP/SMAD signalling. In both animals and humans, mutations in TMPRSS6 result in elevated hepcidin expression and defective iron absorption, which consequently lead to iron-deficiency anaemia (Delbini et al., 2010). Studies that identified TMPRSS6 variants associated with haematological parameters, suggest that TMPRSS6 is crucial in the control of iron homeostasis and normal erythropoiesis (Cau et al., 2010). It has been demonstrated that the treatment of mouse models of HH (*Hfe*^{−/−}) and β -thalassaemia intermedia with TMPRSS6 siRNA formulated in lipid nanoparticles increased hepcidin expression and lessened iron loading in both experimental models (Schmidt et al., 2013).

10.1.3. BMP6 analogues and small molecule activators of STAT/SMAD pathways

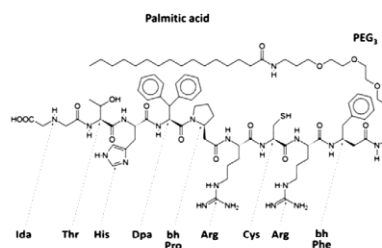
BMP6–SMAD signalling is one of the main regulatory mechanisms in the control of hepcidin expression. HFE interacts with the BMP6–SMAD signalling pathway, downstream of BMP6, to regulate hepcidin expression (Corradini et al., 2010). One technical challenge is to generate antibodies that can specifically recognize BMP6 without significant cross-reactivity to other BMPs. BMP6-like agonists may have a role to play as a new concept in therapeutic strategy.

A small-scale chemical screening study in zebrafish embryos identified the isoflavone genistein as an enhancer of hepcidin transcription (Zhen et al., 2013). Genistein is a soy-derived biologically active isoflavone that exhibits diverse health-promoting effects. A growing body of evidence shows that genistein presents pleiotropic effects. It influences lipid homeostasis and insulin resistance, and counteracts inflammatory processes (Nagaraju et al., 2013). The treatment of HepG2 cells with genistein increased both hepcidin transcript levels and promoter activity. The effect of genistein on hepcidin expression did not depend on increased cellular iron uptake, but was impaired by mutation of

Table 1

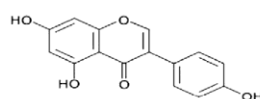
Direct and indirect hepcidin agonists and antagonists.

Direct and indirect hepcidin agonists
Mini-hepcidin
P65



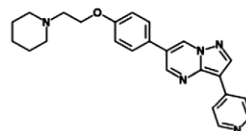
Monoclonal antibodies
LY2787106
Serine protease
TMPRSS6
BMP6 analogues
Genistein

811-AA human protein

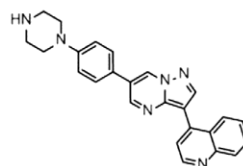


Anticalins
PRS-080
PRS-110
Spiegelmers
NOX-H94
NOX-H94002

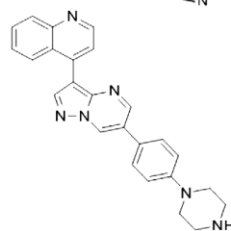
Inhibitors of hepcidin production
Targeting the BMP-HJV-SMAD pathway
–Dorsomorphin



–Dorsomorphin derivatives
LDN-193189



DM-3189



Anti-cytokine agents
–anti-TN α antibody
Golimumab
STAT3 inhibitors
–AG 490

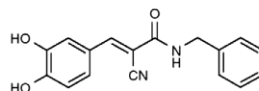
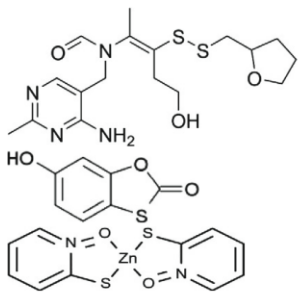


Table 1 (continued)

–PpYLKTL	H-Pro-Tyr-(PO ₃ H ₂)-Leu-Lys-Thr-Lys-Ala-Ala-Val-Leu-Leu-Pro-Val-Leu-Leu-Ala-Ala-Pro-OH
Pharmacological compounds with hepcidin antagonistic properties	
Fursultiamin	
Thioxolone	
Pyrimithione zinc	
Vitamin C	
Vitamin A	
Vitamin D	
Statins	

either BMP response elements or the STAT3-binding site in the hepcidin promoter (Zhen et al., 2013). Genistein and other candidate molecules may subsequently be developed into new therapies for iron-overload syndromes.

10.2. Direct and indirect hepcidin antagonists

10.2.1. Antibodies, short interference RNA and antisense oligonucleotides against hepcidin

10.2.1.1. Monoclonal antibodies. Strategies have been developed that neutralize hepcidin activity by directly binding to the hepcidin peptide through monoclonal antibodies (mAbs), engineered protein and RNA-based binders. High-affinity antibodies specific for human hepcidin (hHepc) have been generated, and hHepc knock-in mice have been produced. Hepcidin mRNA suppression or antibody-mediated neutralization was able to overcome inflammation-induced anaemia in a mouse model (Sasu et al., 2010). RNA interference (RNAi) and gene-silencing antisense oligonucleotides that target transcription or translation of hepcidin are other approaches to develop therapeutic strategies for inflammation-induced anaemia. Antisense oligonucleotides that inhibit the translation of hepcidin or its regulators, such as HJV, are currently in the discovery stages of development.

Surface markers, such as CD2, CD3, CD4, CD25, and CD52, expressed on T-cells have been discovered and are now specific therapeutic targets. CD52 mAb targets the cell surface CD52 and is effective in depleting lymphocytes through cytolytic effects *in vivo*. The aim of a recent study was to investigate the therapeutic effect of anti-mouse CD52 mAb on Fe-deficient anaemia in inflammatory bowel disease. The results indicated that anti-CD52 therapy may ameliorate Fe-deficient anaemia (Wang et al., 2014). Human or humanized mAbs against hepcidin have been developed by scientists at Lilly (US Patent 7820163) and Amgen (US Patent Application 12/022515). To examine the contribution of hepcidin to inflammatory anaemia, and the effect of mAbs, a mouse model of inflammation-induced anaemia was required (Sasu et al., 2010). A humanized monoclonal antibody against hepcidin is in a phase I human trial. This study will evaluate the safety of LY2787106 in patients with cancer and anaemia.

10.2.1.2. Anti-BMP6 monoclonal antibody and antisense oligonucleotides. Anti-BMP6 monoclonal antibody therapy is another option to specifically block BMP6-mediated hepcidin regulation. The administration of

anti-BMP mAb in healthy mice decreased hepatic hepcidin expression and increased serum iron levels (Andriopoulos et al., 2009). In the Hfe transgenic mouse model of excess hepcidin and iron-deficiency anaemia, treatment with anti-BMP6 improved the anaemia by lowering hepcidin levels (Corradini et al., 2010).

Antisense oligonucleotides that inhibit the translation of hepcidin or its regulators such as HJV are in the discovery stages of development. Systemic delivery of antisense molecules results in preferential delivery to the liver, making them possible agents to target hepcidin (Graham et al., 1998). RNA-based targeting of hepcidin regulators, such as HJV antisense oligonucleotides and TIR2 siRNA, is under development. Treatment with TIR2 siRNA potentially decreased hepcidin mRNA in a rat model of inflammation-induced anaemia (Querbes et al., 2012).

10.2.2. Anticalins: specific anti-hepcidin and Spiegelmer-based hepcidin

Anticalins are a novel class of compounds, with applications in hepcidin pharmacology. They are engineered derivatives of naturally occurring binding proteins called lipocalins. Lipocalins are mediators in a context of inflammation. They present high target specificity, good tissue penetration and low immunogenicity. They are involved in the transport of small hydrophobic molecules such as steroids and lipids into the cell. Due to their compact molecular architecture (they comprise a single polypeptide chain), they could be of interest as a therapy (Gebauer & Skerra, 2012). Recently, anticalin PRS-080, which specifically binds human hepcidin with sub-nanomolar affinity, has been described. It is a selective specific inhibitor that blocks the interaction between hepcidin and the FPN receptor. Significant dose-dependent and hepcidin-specific iron mobilization was observed in Cynomolgus monkeys, following a single 3 mg/kg *i.v.* bolus of PRS-080. A programme is currently under way to organize the first human clinical trial.

Different Spiegelmer-based hepcidin binders have now been identified. Spiegelmers are L-enantiomeric oligonucleotides that can be designed to inhibit pharmacologically relevant target molecules. They bind to the targets in a manner comparable to antibodies. Given their non-natural, mirror-image nature, Spiegelmers are nuclease-resistant and immunologically passive. Spiegelmers can fold into distinct three-dimensional structures that generate high-affinity ligands (Klussmann et al., 1996). The best binding Spiegelmer, NOX-H94, has been modified to the L-oligonucleotide itself (NOX-H94002) and its PEGylated variant NOX-H94. NOX-H94 is a structured mirror-image L-oligonucleotide that binds human hepcidin with high affinity, thereby blocking its biological function (Schwoebel et al., 2013).

10.2.3. Inhibitors of hepcidin production

Inhibition of hepcidin is a potential therapeutic approach to improve the utilization of iron from intracellular stores in patients suffering from anaemia of chronic inflammation.

10.2.3.1. Targeting the BMP6–HJV–SMAD pathway. As the BMP pathway plays a key role in stimulating hepcidin transcription, sequestration of BMP ligands could decrease hepcidin expression. Heparin, a glycosaminoglycan widely used as an anticoagulant, is known to bind BMPs. The treatment of mice with pharmacological doses of heparin inhibited liver hepcidin mRNA expression and SMAD phosphorylation, reduced spleen iron concentrations, and increased serum iron (Poli et al., 2011). Heparins are efficient hepcidin inhibitors both in vitro and in vivo, but their anticoagulant activity limits their therapeutic use. Non-anticoagulant heparins, produced by N-acetylation and oxidation/reduction (glycol-split) that abrogates antithrombin binding affinity, have recently been studied. These non-anticoagulant compounds impair BMP/SMAD signalling with no evident adverse effect in vivo (Poli et al., 2014). They may offer a strategy for the treatment of diseases with high hepcidin levels. Inhibition of BMP type I receptor signalling by small molecule inhibitors was also effective in moderating iron-restricted anaemia. Through a structure–activity relationship study of dorsomorphin derivatives, optimized compounds (LDN-193189 or DM-3189) with higher activity and specificity on BMP type I receptors have been also developed (Cuny et al., 2008). LDN-193189 lowers hepcidin levels, mobilizes spleen iron stores, increases serum iron and iron incorporation into reticulocytes (Sun et al., 2013).

Concerning these compounds derived from dorsomorphin, various important off-target effects persist. Programmes that aim to identify selective modulators have been developed. The challenge lies in the synthesis of potent and biologically available selective inhibitors of BMP type I receptor kinases. In this domain, 4-quinolinylnyl substituted dorsomorphin helped to highlight novel roles of this receptor system in pathophysiological processes (Hong & Yu, 2009).

Evidence of interplay between cholesterol and iron metabolism, has been demonstrated in macrophages. Data suggest that reducing intracellular free iron levels within macrophages by increasing the expression of macrophage FPN may be a promising strategy to increase the expression of cholesterol efflux transporters (Saeed et al., 2012). LDN-193189, a small molecule inhibitor of BMP signalling, has been studied. Serum iron levels were significantly higher following treatment with LDN-193189. Peritoneal macrophages isolated after treatment with LDN-193189 showed reduced intracellular iron and hydrogen peroxide production. To evaluate the effect on atherosclerosis, ApoE^{−/−} mice on a high-cholesterol diet were treated with LDN-193189. Significant reductions were found in intraplaque Oil Red O-positive lipid area, total plaque area, and plaque severity, along with elevated ATP binding cassette transporter A1 (ABCA1) immunoreactivity within plaque macrophage-rich regions. These findings suggest that LDN-193189 increases the expression of ABCA1 in macrophages within atherosclerotic plaques, thus limiting plaque progression.

Soluble HJV-Fc fusion protein (sHJV-Fc) is another agent that prevents the interaction between BMPs and their receptors. sHJV is known to bind BMP6, and this results in decreased SMAD signalling in the cell and decreased hepcidin expression. sHJV is a soluble fragment of the membrane-linked BMP co-receptor HJV, and the soluble and membrane form have opposing effects on hepcidin expression. In this context, the pharmacological inhibition of hepcidin expression via this pathway results in the mobilization of iron from the reticulo-endothelial system, stimulation of erythropoiesis and the correction of anaemia (Theurl et al., 2011).

Alcohol consumption has long been associated with changes in iron homeostasis. Anaemia and iron deficiency can be due to gastrointestinal blood loss arising from the complications of alcohol abuse. Iron-mediated cell signalling has been reported to be involved in the pathogenesis of experimental alcoholic liver disease (Harrison-Findik, 2009).

The mechanism was recently shown to involve inhibition of the BMP–SMAD pathway. The inhibition of BMP receptor activation and signalling by alcohol may involve various mechanisms such as hypoxia in the liver and inhibition of SMADs binding to hepcidin promoter (Gerjevic et al., 2012).

10.2.3.2. Anti-cytokine agents. Another targeting pathway is via anti-cytokine agents, since hepcidin expression is strongly induced by IL-6/STAT3. The benefit of these agents used to treat inflammatory diseases may be provided by reducing hepcidin expression. Anti-TNF α antibody (golimumab) in patients with rheumatoid arthritis is associated with a continuous decrease in hepcidin levels (Doyle et al., 2013).

Targeting the erythropoietic pathway may also be a pharmacological strategy. A single injection of erythropoietin (EPO) in human volunteers results in a rapid reduction of serum hepcidin (Ashby et al., 2010). Erythropoiesis-stimulating agents such as prolyl hydroxylase inhibitors have been tested in clinical trials. The increased production of EPO was able to lower hepcidin. Unfortunately, however, these biological effects were associated with major detrimental disorders (Schwoebel et al., 2013).

10.2.3.3. STAT3 inhibitors. The high level of hepcidin expression linked to the STAT3 pathway led to the testing of STAT3 inhibitors. Treatment with small molecule STAT3 inhibitors such as AG490 and the synthetic peptide inhibitor of STAT3 (PpYKTK) abolished hepcidin expression in mice. AG490 inhibits the phosphorylation of STAT3 by JAK2, while the PpYKTK disrupts phospho-STAT3 dimerization, which is required for binding target genes (Fatih et al., 2010). In anaemia, AG490 could be a possible strategy to attenuate hepcidin-mediated biological actions in order to enhance iron uptake through enterocytes and iron release from macrophages. Another STAT3 inhibitor is curcumin. It has been proposed as a therapeutic adjuvant during inflammation, and its potential iron chelating activity has been reported (Aggarwal & Shishodia, 2006). Another way to inhibit hepcidin production is associated with blocking antibodies to the IL-6 ligand. This strategy resulted in hepcidin lowering effects. Siltuximab, previously known as CNTO 328, is a chimeric, immunoglobulin (Ig) G κ mAb that binds and neutralizes human IL-6 with high affinity and specificity with potential therapeutic effects (van Rhee et al., 2010).

10.2.3.4. Pharmacological compounds with hepcidin antagonistic properties. One group of compounds with hepcidin antagonistic properties are cardiac glycosides. Nanomolar concentrations of cardiac glycosides prevent the internalization of FPN. These concentrations are much lower than those known to cause adverse effects (Riganti et al., 2011).

Small molecules identified as hepcidin antagonists such as fursultiamin, thioxolone, and pyridine zinc are FDA-approved compounds that contain a sulphur moiety with potential thiol-directed chemical reactivity. These compounds prevent the interaction between hepcidin and FPN. Fursultiamin, a vitamin B1 derivative, is the thiamine derivative, thiamine tetrahydrofurfuryl disulphide (TTFD), which is absorbed from the intestinal tract and converted into thiamine pyrophosphate (TPP). It plays an important role as a cofactor for enzymes of the metabolic pathways involved in the production of adenosine triphosphate (ATP). Because fursultiamin prevents hepcidin-mediated endocytosis of FPN, it is hypothesized that fursultiamin may prevent FPN ubiquitination and thus interfere with the hepcidin/FPN interaction (Fung et al., 2013).

Recent advances in our understanding of the molecular mechanisms of iron regulation reveal that the production of hepcidin may be regulated by the vitamin environment. As we reported previously, in conditions of vitamin D deficiency, elevated synthesis of hepcidin by hepatocytes may increase intracellular and systemic concentrations of hepcidin and decrease membrane expression of FPN in these cells. On the other hand, iron bioavailability seems to be regulated by antioxidant vitamins. Correlations between biomolecule oxidation products and

iron status biomarkers have been observed in numerous studies (Cottin et al., 1998; de Valk & Marx, 1999). It has been proposed that hepcidin was able to protect tissue by inhibiting lipid peroxidation through indirect processes involving iron metabolism. The relationship between antioxidants and hepcidin mobilization appears complex given the versatility of iron as an oxidizing/reducing agent. The relationship between an antioxidant such as vitamin C and hepcidin expression has not yet been clearly explained. However, it is known that in patients with chronic renal failure, treatment with high doses of EPO or vitamin C can correct the functional iron deficiency (Pinto et al., 2008). In a human liver carcinoma cell line (HepG2), it has been reported that vitamin C directly inhibits hepcidin expression (Chiu et al., 2012). Concerning the effect of vitamin A deficiency on iron status; vitamin A metabolites have been shown to affect the regulation of the synthesis and catabolism of proteins involved in iron metabolism. Vitamin A deficiency increased liver hepcidin mRNA levels (Schroeder et al., 2007; Arruda et al., 2009).

A number of studies have highlighted the association between increased inflammatory indices and a reduced response to erythropoietin-stimulating agents (ESAs). It has been suggested that statins may have a positive impact on ESA responsiveness. A recent study showed that simvastatin significantly suppressed the mRNA expression of hepcidin in HepG2 cells (Chang et al., 2013). Statins, which are inhibitors of 3-hydroxy-3-methyl glutaryl co-enzyme A reductase, not only reduce lipid levels, but also have numerous pleiotropic effects. In fact, the precise molecular mechanisms responsible for changes in hepcidin mRNA expression are still unclear (Ha et al., 2009). Statin therapy may improve responsiveness to erythropoietin-stimulating agents in patients with end-stage renal disease. Recent studies suggest that statins increase the erythropoiesis response by targeting hepcidin and iron regulation pathways, independently of erythropoietin (Chang et al., 2013). One possible interpretation of these results is that the antioxidant properties of statins may be implicated in these effects (Sicard et al., 2007, 2008).

11. Hepcidin: an endogenous cytoprotective agent in cardiovascular pathophysiology and possible therapeutic targets?

The presence of hepcidin in body fluids raises questions about its function within these compartments. The role of hepcidin as an antimicrobial agent is well established (Verga Falzacappa & Muckenthaler, 2005) and given that body fluids play an important role in the natural defences against inflammation, it is conceivable that hepcidin plays a role in cellular protection.

It is now well established that LPS mediates its effects through TLR4, for the induction of pro-inflammatory genes. LPS, potent inducers of pro-inflammatory cytokines, are responsible for endotoxemic shock (Lecour et al., 2002). Endotoxemic shock is associated with a pro-inflammatory response and hypoferrremia. Hepcidin has emerged as the possible pathogenic mediator of hypoferrremia (Paya et al., 1995). In cholestatic mice, pre-treatment with hepcidin reversed the adverse effects of LPS administration and thus reduced early mortality. In this experimental model, pre-treatment with recombinant hepcidin significantly reduced LPS-induced pro-inflammatory cytokines and hepatocellular injury (Huang et al., 2012).

Myocardial protection aims to prevent reversible post-ischemic cardiac dysfunction and irreversible myocardial cell death (myocardial infarction) that occur as a consequence of myocardial ischemia and/or ischemic-reperfusion injury. Cardiopulmonary bypass (CPB) and the necessary period of ischemia-reperfusion are situations in cardiac surgery that promote oxidative stress. Reperfusion injury occurs, for example, after coronary artery bypass grafting when the ischemic myocardium is again provided with a supply of blood. Its most serious manifestations are arrhythmia and myocardial stunning, which are associated with free radical production (Clermont et al., 2002; Goudeau et al., 2007). The role of iron in reperfusion injury has been shown by indirect evidence: during reperfusion syndrome, the binding of iron to the

chelator desferrioxamine protects the myocardium subjected to ischemia-reperfusion. The significant decrease in apoptosis in hearts treated with desferrioxamine could be considered evidence of a close link between iron chelation, oxidative stress and the promotion of apoptotic death in ischemia-reperfusion injury (Dobsak et al., 2002; Galaris et al., 2006). As we previously reported, hypoxia resulted in the strong up-regulation of hepcidin mRNA and protein expression in the heart. The regulated expression of hepcidin in the heart suggests that hepcidin may play an important role in the development of cardiac diseases (Merle et al., 2007). CPB is a convenient and highly relevant model to study novel biomarkers of acute kidney injury (AKI). Urinary hepcidin has been suggested as a candidate biomarker of AKI (Ho et al., 2009). In a clinical study, the relationship between changes in serum hepcidin, urinary hepcidin concentrations, and the urinary hepcidin/creatinine ratio were examined in a cohort of patients undergoing cardiothoracic surgery with CPB. Urinary hepcidin and the hepcidin/creatinine ratio were shown to be biomarkers of AKI after CPB, with an inverse association between its increase at 24 h and the risk of AKI in the first five post-operative days (Prowle et al., 2012). In a recent study, it was reported that serum hepcidin and urinary hepcidin increased immediately post-operatively and at 24 h (Haase-Fielitz et al., 2013). In this study of 100 patients undergoing cardiac surgery, low preoperative urine and plasma hepcidin concentrations were predictors of in-hospital mortality. These results suggest that hepcidin plays an important role in modulating and determining the course and outcome of inflammation-mediated diseases.

Chronic heart failure (CHF) is a cardiovascular disease with a high rate of mortality. Anaemia is a common comorbidity in these patients, and is associated with a worse prognosis. However, the pathophysiology of anaemia in these patients is still not very well understood and is thought to be a complex and multifactorial process (Anand, 2008). Patients with CHF who became anaemic had higher levels of hepcidin (Martinez-Ruiz et al., 2012).

Finally, as mentioned above, several reports suggest that hepcidin concentrations could be used to determine cardiovascular risk. It has been hypothesized that increased hepcidin concentrations may aggravate cardiovascular risk by modifying intracellular iron concentrations of macrophages and by increasing their atherogenic potential (Lapenna et al., 2007).

12. Conclusion

Iron is the only micronutrient known to have a regulatory hormone, hepcidin, that responds to both nutrient status and infections. Hepcidin acts to block both iron absorption in the gut and iron release from macrophages through a common mechanism. The discovery of the hormonal iron-regulating role of hepcidin, followed by the elucidation of its mechanism of action has led to better understanding of the pathophysiology of human iron disorders. Positive regulators of hepcidin, which lead to reduced ferroportin and decreased availability of iron, include increased circulating iron and inflammatory cytokines. Measuring levels of hepcidin could be useful in diagnosing several cardiovascular dysfunctions associated with haematological diseases. Iron-overload diseases are typically insidious and cause progressive and irreversible organ injury before clinical symptoms appear. In addition, better understanding of the relationship between the structure and function of hepcidin and the hepcidin-FPN complex may lead to the design of hepcidin antagonists and agonists for human therapy. The development of research in this domain offers new clinical potential in terms of diagnosis and therapy.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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2. Fer, stress oxydant et signalisation redox dans le système cardiovasculaire

Si les phénomènes oxydatifs sont la base d'un certain nombre de pathologies, la thérapie évidente pour celles-ci devrait être l'utilisation d'antioxydants. Cependant, aucun avantage clair des traitements anti-oxydants n'a à ce jour été démontré dans des essais cliniques [144]. En revanche, il semble que le fer joue un rôle central dans la formation et le piégeage des espèces réactives de l'oxygène et de l'azote. Le fer est un déterminant majeur de l'oxydoréduction cellulaire. Il participe à la réaction de Fenton comme donneur d'électron au peroxyde d'hydrogène, aboutissant à la formation d'une espèce radicalaire puissante, le radical hydroxyle. Ce métal est également impliqué dans des processus catalytiques qui découlent de sa réactivité redox, qui lui permet de faire la transition entre un état ferreux réduit et un état ferrique oxydé. La compréhension des mécanismes associés au développement d'une dysfonction de l'homéostasie du fer semble indispensable. Il est de plus d'une importance cruciale de comprendre les mécanismes précis expliquant comment les différents compartiments du fer peuvent être impliqués dans le stress oxydatif local. Les chélateurs du fer pourraient représenter une nouvelle approche pour interrompre le cycle du stress oxydant induit par le catabolisme du fer et les lésions tissulaires [145, 146]. Les chélateurs du fer pourraient apporter un bénéfice thérapeutique dans de nombreuses conditions pathologiques. Des avancées ont été réalisées dans des essais précliniques et cliniques sur l'intérêt d'un traitement chélateur du fer pour le traitement de la surcharge en fer mais aussi pour les maladies chroniques inflammatoires et cardiovasculaires. Nous avons étudié les interactions entre le fer, le stress oxydant et la signalisation redox dans le système cardiovasculaire dans une troisième revue publiée en 2014 dans *Molecular Nutrition and Food Research* (85).

REVIEW

Iron, oxidative stress, and redox signaling in the cardiovascular system

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The redox state of the cell is predominantly dependent on an iron redox couple and is maintained within strict physiological limits. Iron is an essential metal for hemoglobin synthesis in erythrocytes, for oxidation–reduction reactions, and for cellular proliferation. The maintenance of stable iron concentrations requires the coordinated regulation of iron transport into plasma from dietary sources in the duodenum, from recycled senescent red cells in macrophages, and from storage in hepatocytes. The absorption of dietary iron, which is present in heme or non-heme form, is carried out by mature villus enterocytes of the duodenum and proximal jejunum. Multiple physiological processes are involved in maintaining iron homeostasis. These include its storage at the intracellular and extracellular level. Control of iron balance in the whole organism requires communication between sites of uptake, utilization, and storage. Key protein transporters and the molecules that regulate their activities have been identified. In this field, ferritins and hepcidin are the major regulator proteins. A variety of transcription factors may be activated depending on the level of oxidative stress, leading to the expression of different genes. Major preclinical and clinical trials have shown advances in iron-chelation therapy for the treatment of iron-overload disease as well as cardiovascular and chronic inflammatory diseases.

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1 Introduction

The term “oxidative stress” is frequently used in a number of biochemical, physiological, and pathophysiological situations. It describes the result of increased reactive species production and/or a decrease in their elimination. Indeed, aging is the result of a progressive vicious spiral in which oxidative stress plays a major role. Oxidative stress has been identified as critical in most of the key steps in the pathophysiology of cardiovascular diseases. Whether the cell is considered a unicellular organism or a component of organ tissue, it is possible to distinguish various intracellular sources of free radicals. The generation of reactive oxygen species (ROS) is significantly enhanced in the failing myocardium, as has been unequivocally shown by studies of human patients [1–3]. A large body of evidence shows the involvement of cellular free radicals in the redox regulation of normal physiological functions as well as the pathogenesis of various diseases [4]. Oxidative stress is associated with an imbalance of prooxidant factors and direct and/or indirect antioxidants. Thus, cells have developed an elaborate intracellular antioxidant defense system to protect themselves from oxidative damage. The redox state of the cell is predominantly dependent on

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Abbreviations: AF, atrial fibrillation; ALA, 5-aminolevulinic acid; ALAS, ALA synthase; AMI, acute myocardial infarction; CHF, chronic heart failure; CO, carbon monoxide; CRT, cardiac resynchronization therapy; DAMPs, damage-associated molecular patterns; DMT1, divalent metal transporter 1; EPO, erythropoietin; ESA, erythropoietin-stimulating agent; FPN1, ferroportin 1; FRDA, Friedreich's ataxia; GPx, glutathione peroxidase; GSH, reduced glutathione; GSSG, oxidized glutathione; HIF, hypoxia-inducible factor; HO, heme oxygenase; ICD, immunogenic cell death; ID, iron deficiency; IRPs, iron regulatory proteins; Keap1, Kelch-like ECH-associated protein 1; MI, myocardial infarction; NADPH, nicotinamide adenine dinucleotide phosphate; NF- κ B, nuclear factor kappa B; NO, nitric oxide; NOS, nitric oxide synthase; NOX, nicotinamide adenine dinucleotide phosphate oxidase; Nrf2, nuclear factor erythroid-related factor 2; PLC, phospholipase C; RNS, reactive nitrogen and oxygen species; ROS, reactive oxygen species; SOD, superoxide dismutase; SR, sarcoplasmic reticulum; Tf, transferrin; Tfr, Tf receptor; TLR, toll-like receptor; Trx, thioredoxin

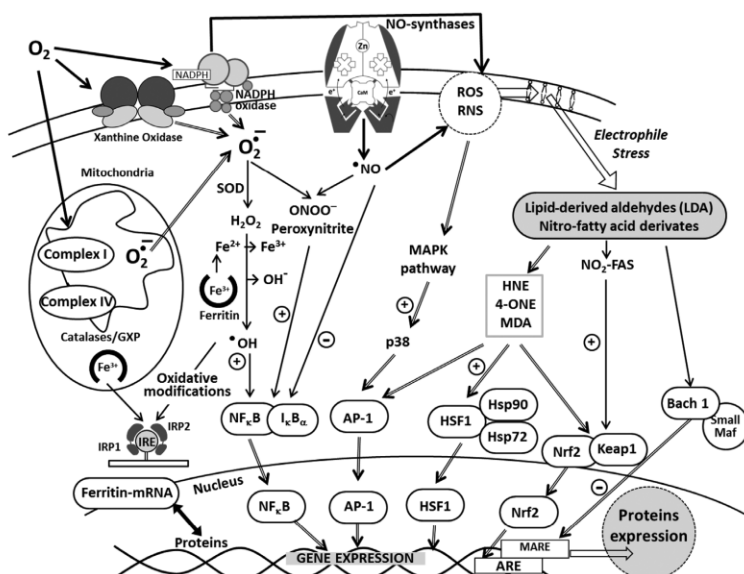


Figure 1. Diagram showing elements of the unifying mechanisms of adaptive response to oxidative and electrophile stress; oxidative modifications associated with iron metabolism. ROS, reactive oxygen species; RNS, reactive nitrogen species; endogenous and exogenous electrophiles/activators (see text for more details) can alter transcription factors and signaling complexes (AP-1, HSF1, NF- κ B/I- κ B, Nrf2-Keap1, and Bach-1). Subsequent nuclear translocation and the induction of ARE-driven genes result in upregulated environmental stress response and protein expression. IRP1 and IRP2 regulate expression of iron metabolism genes. The regulation of ferritin synthesis by iron is mainly due to posttranscriptional regulation through the binding of IRP1 and IRP2 to an iron-responsive elements (IRE) located in the 5' UTR of ferritin mRNA.

an iron (and copper) redox couple and is maintained within strict physiological limits [5]. Within the cells, iron is in a dynamic equilibrium mainly between four compartments as follows: vesicular iron, the labile iron pool, functional iron, and storage iron [6]. It is of pivotal importance to understand (1) the possible mechanisms of how different iron compartments may be implicated in local oxidative stress and (2) the spatial and temporal constraints of reactive species signaling, and the functional role of the antioxidant responses.

2 Sources and metabolism of reactive nitrogen and oxygen species (RNOS) and redox signaling

2.1 ROS and reactive nitrogen species (RNS)

The cellular (reduction–oxidation) redox environment refers to the reduction potential or reducing capacity in cellular compartments (Fig. 1). Cellular redox status is regulated by the balance between cellular oxidant and reductant levels. Oxidative and reductive stress can trigger redox cascades and affect the environment of the cell, for example, exposure to various xenobiotics might determine if a cell will proliferate, differ-

entiate, or die [7]. Free radicals have emerged as important regulators of many physiological and pathological processes through redox processes. High levels of free radical production that overwhelm cellular antioxidant defense systems may damage biomolecules, and deregulate signaling pathways. Oxidative stress occurs during a profusion of pathological processes, including cardiovascular disease, atherosclerosis, diabetes, inflammation, and apoptosis [8].

2.1.1 Definition and sources of ROS and RNS

Free radicals can be defined as molecules or compounds that contain one or more unpaired electron thus making them highly reactive. Radicals derived from oxygen (ROS) and nitrogen (RNS: derived from nitric oxide [NO]) are the largest class of radical species generated in living systems; ROS and RNS are collectively referred to as RNOS. RNOS are products of cell metabolism and have either beneficial or deleterious effects, depending on the concentration reached in the area of the cells [9, 10]. RNOS include superoxide ($O_2^{\cdot-}$), the hydroxyl radical ($\cdot OH$), carboxyl radical ($CO_2^{\cdot-}$), $\cdot NO$, and ($\cdot NO_2$) as well as the nonradical species hydrogen peroxide

(H₂O₂), hypochlorous acid (HOCl), singlet oxygen, and carbon monoxide (CO).

Several cell sources produce ROS under normal physiological conditions, including the mitochondrial electron transport chain, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX), cytochrome P450, lipoxygenases, cyclooxygenases, xanthine oxidases, peroxisomal enzymes, and, under certain conditions, endothelial NO synthases (NOSs) [11]. Five multiprotein complexes constitute the respiratory chain embedded in the inner membrane of the mitochondria. Complexes I and III are the main sites of O₂^{•-} generation. An important source of ROS produced in endothelial cells is from oxidases (xanthine oxidases, NADPH oxidases). Xanthine oxidase catalyzes the sequential hydroxylation of hypoxanthine to yield xanthine and uric acid. The enzyme exists in two forms that differ primarily in their oxidizing substrate. The dehydrogenase form is preferentially used. Activation of NADPH oxidases may result from the stimulation of a number of cell surface receptors, such as the angiotensin II receptor, which is particularly important in hypertension and heart failure. Upon stimulation by angiotensin II, the activity of NADPH oxidases is increased in endothelial and smooth muscle cells, suggesting that in the presence of an activated renin–angiotensin system (either local or circulating), dysfunction due to increased vascular production of superoxide anions is to be expected [12, 13]. The stimulation of angiotensin receptor 1 by angiotensin II may lead to phospholipase C and D activation, both of which produce diacylglycerol. Phospholipase C also produces inositol triphosphate. Diacylglycerol and IP₃-mediated calcium release activates protein kinase C [14]. Phosphorylation of the p47phox subunit by protein kinase C is required to enable binding of this subunit with others and thus to activate NADPH oxidases.

The major RNS is endothelium-derived 'NO. Under physiological conditions, in the presence of substrate (L-arginine) and cofactors (tetrahydrobiopterin) endothelial NOS produces 'NO. It is a potent gaseous mediator widely accepted as a key determinant of endothelial function produced by endothelial NOSs. Enzymatic sources of ROS implicated in disease are uncoupled NOSs (which generate injurious superoxide rather than vasoprotective NO), xanthine oxidoreductase, mitochondrial respiratory enzymes, and NADPH oxidase. Of the many ROS-generating enzymes, NADPH oxidase, of which there are seven homologues (termed Nox 1–5, Duox1,2), appears to be particularly important in cardiovascular disease [15]. NO has potent vasodilator, anti-inflammatory, and anti-thrombotic properties [16, 17]. The free radical 'NO has a half-life of only a few seconds in an aqueous environment. 'NO reacts with molecular oxygen and ROS to generate a range of oxidation products. One well-characterized RNS-forming reaction is that of 'NO with O₂^{•-}. This reaction occurs at nearly diffusion-limited rates to produce peroxynitrite, ONOO⁻, which itself is strongly oxidizing, and when protonated, undergoes homolytic scission to produce hydroxyl radical ('OH) and nitrogen dioxide ('NO₂). Additional reac-

tive radicals derived from other endogenous molecules such as CO and hydroperoxyl can be formed in living systems.

2.1.2 Controls of ROS and RNS: enzymatic and nonenzymatic components

The rigorous control of ROS levels is essential for cell survival owing to the toxicity of ROS at high levels. Elaborate intracellular antioxidant defense systems exist in the different parts of the cells to protect cells from oxidative damage. An antioxidant can be defined as any substance that when present in very low concentrations compared to that of an oxidizable substrate significantly delays or inhibits the oxidation of that substrate. A complex network of antioxidants includes both enzymatic and nonenzymatic components that regulate ROS production within the cell, both spatially and temporally. The transcriptional control of the antioxidant enzyme system is another key mechanism in the maintenance of cell redox homeostasis. Normal cells maintain redox homeostasis with low basal ROS levels and have spare antioxidant capacity to cope with higher levels of ROS and RNS. The antioxidants can be endogenous or exogenous, for example, ingested in the diet or as dietary supplements. Some dietary compounds that do not neutralize free radicals, but enhance endogenous activity may also be classified as antioxidants. There is intense interest in flavonoids due to their antioxidant and chelating properties and their possible role in the prevention of chronic and age-related diseases.

An endogenous scavenger, such as glutathione, exists in both reduced (GSH) and oxidized (GSSG) states. The balance between GSH/GSSG and pyridine nucleotides (NADH/NAD, NAD(P)H/NADP) determines the cellular redox status and the level of oxidative stress [18]. Nonenzymatic antioxidants can be classified further into directly acting antioxidants (e.g., scavengers and chain-breaking antioxidants) and indirectly acting antioxidants (e.g., chelating agents). Antioxidants scavenge free radicals, thus preventing free radical chain reactions. Antioxidant vitamins (i.e., vitamins C, E, A, and folic acid) are involved in some of the main defense mechanisms of the body's nonenzymatic antioxidant systems. Several other antioxidants (*N*-acetylcysteine, lipoic acid, and glutathione) are able to reduce lipid peroxidation products [19–21]. Antioxidant defense enzymes, such as heme oxygenase 1 (HO-1), catalase, superoxide dismutase (SOD), peroxiredoxin, and glutathione reductase, are able to cause a significant decrease in lipid peroxidation products. The vascular wall also contains these various enzymes that can reduce the ROS burden [22].

Oxidative stress is able to induce deleterious modifications in a variety of cellular compounds: DNA, lipids, and proteins. ROS can attack endogenous molecules indirectly by reacting with cellular membrane phospholipids, resulting in the generation of secondary reactive intermediates. Phospholipids are present at high concentrations within PUFA residues and have been identified as prime targets for oxidation.

Malondialdehyde and 4-hydroxynonenal are the major products of lipid peroxidation (Fig. 1) [23]. Malondialdehyde is suggested to have both mutagenic and carcinogenic effects [24] while 4-hydroxynonenal is proposed to have an effect on cellular signal transduction pathways. Lipids are susceptible to oxidative degradation caused by radicals, and during autoxidation (peroxidation) the chain reaction is mediated by peroxy radicals causing damage to biomembrane integrity. Endogenous antioxidants provide an active system of defense against lipid peroxidation. However, the effectiveness of their antioxidant action depends on several parameters, including localization. In this respect, the functions of endogenous antioxidant vitamins are very important as they are involved in the temporal and spatial monitoring of oxidative metabolic processes. Vitamin E (α -tocopherol) acts as a “chain-breaking” antioxidant in that it forms an α -tocopherol radical that stops the propagation of lipid peroxidation. α -Tocopherol radical can be converted back to vitamin E by the potent antioxidants vitamin C (ascorbic acid) and glutathione [25,26]. Oxidative stress is associated with dysfunction of the mitochondria and ER, inducing apoptosis and protein misfolding. Mitochondria are endowed with an elaborate and well-defined multilevel antioxidant defense system of enzymes and nonenzymes to quench ROS. The scavenging system includes a number of endogenous processes that involve SODs, GSH, and thioredoxin (Trx) systems; peroxidases; and catalases. The major antioxidant enzymes possess transition metals or selenium in their active center. For example, there are three forms of SOD present in humans: cytosolic Cu, Zn-SOD, mitochondrial Mn-SOD, and extra cellular-SOD.

2.2 Transition metals and oxidative stress

Transition metals, including iron, copper, manganese, zinc, and selenium, play a prominent role in cell metabolism. Iron and copper participate in the Fenton reaction as an electron donor to hydrogen peroxide with the subsequent production of a potent ROS species, the hydroxyl radical. Iron is implicated in catalytic processes that stem from its redox reactivity, which enables it to transition between a reduced ferrous and oxidative ferric state [27]. Maintaining cellular iron content requires precise mechanisms to regulate its uptake, storage, and export. The amount of iron absorbed by enterocytes depends on the body's demands. One mechanism involved in iron homeostasis is regulated not only by cellular iron status, but also by ROS in cells that elicit defense mechanisms against iron toxicity and iron-catalyzed oxidative stress [28].

Like iron, copper is a redox-active metal that can transit from a reduced Cu^{1+} form to an oxidized Cu^{2+} state. Copper is critical in many cellular processes, including antioxidant defenses and iron oxidation. Cellular copper levels must also be tightly controlled, as the accumulation of copper can lead to oxidative stress [29]. Manganese is an essential trace metal and cofactor for the mitochondrial antioxidant enzyme Mn-SOD, as well as other enzymes, such as arginine synthase.

MnSOD appears to be an important contributor to cardiac mitochondrial and metabolic functions by reducing mitochondrial oxidative stress [30]. Selenium is an essential nutritional element to mammals, in that it is necessary for the active function of different oxidant enzymes, including glutathione peroxidase (GPx) and Trx reductases. Eight different isoforms of GPx (GPx1–8) have been identified in humans. GPx1 is the most abundant isoform and is present in the cytoplasm of many mammalian tissues where it reduces hydrogen peroxide to water [31]. Redox homeostasis is crucial for cell viability and normal cell function and is maintained by two major cellular antioxidant systems, including the glutathione system and the Trx system. Thioredoxin reductase reduces its major substrate Trx, but can also transfer electrons to low molecular weight compounds. Thioredoxin-dependent peroxidoredoxins are the first-line defense against ROS and ONOO[−] and regulate H_2O_2 -mediated signal transduction. H_2O_2 can act as either a destructive oxidant or a second messenger [32].

2.3 RNOS and mitochondria

The mitochondria are the major source of ROS production within the cell, and high levels of ROS production are probably associated with a variety of pathophysiological conditions. The majority of ROS in the heart appear to come from uncoupling of the mitochondrial electron transport chain at the level of complexes I and III, although the view that mitochondria are a major source of intracellular ROS has been challenged [33]. When ROS production exceeds the capacity of detoxification, oxidative damage to proteins, DNA (mtDNA and nDNA), and membrane lipids occurs [34]. Each mitochondrion has two specialized membranes the outer mitochondrial membrane and the inner mitochondrial membrane that encloses the matrix. There is a narrow intermembrane space between these two membranes. The outer mitochondrial membrane is a major source of ROS in particular by complexes I and III and monoamine oxidases (mostly the A isoform), thus contributing to the ROS generated by mitochondria under pathological conditions. Under physiological conditions, the production of ROS is estimated to account for about 2–5% of the total oxygen uptake by the organism [35].

Phospholipids, and especially cardiolipin, are crucial for mitochondrial energy metabolism. Indeed, among the specific phospholipids, cardiolipin is known to provide essential structural and functional support to several proteins involved in oxidative phosphorylation [36]. Cardiolipin also plays an active role in mitochondria-dependent apoptosis by interacting with cytochrome c. Cardiolipin and cytochrome c can form a complex called cytochrome c–cardiolipin peroxidase that under oxidative stress can lead to the release of cytochrome c, a proapoptotic factor.

Oxidative stress accompanied by calcium overload and ATP depletion induces mitochondrial permeability transition with the formation of pathological, nonspecific mitochondrial permeability transition pores in the mitochondrial inner

Table 1. Nonheme and heme sources of iron

Nonheme iron food source			Heme iron food source		
Food	ng/100 g	%DV	Food	ng/100 g	%DV
Chocolate (dark)	17	97	Lamb liver	23	129
Parsley	10	55	Oysters	6.6	37
Soybeans	9	50	Beef liver	6.1	34
Lentils	9	50	Beef lean only	3.6	20
Beans navy	7.5	41	Beef 85% lean	2.6	14
Beans black	6.7	37	Chicken	1.1	6
Grapes (dry) seedless	3.3	18	Tuna	0.9	5
Dandelion	3.2	17	Crab	0.9	5
Spinach	2.7	15	Pork	0.8	5
Bread (white)	2	11	Shrimp	0.3	2

DV, daily value.

DVs are reference numbers developed by the Food and Drug Administration (FDA).

membrane [37]. It has been demonstrated that a specific NOS (mitochondria NOS) was localized in cardiac mitochondria [38]. NO has multiple targets in mitochondria, including metalloproteins containing iron–sulfur (Fe–S) clusters that are highly sensitive to direct oxidative modification by NO [39]. The ability of mitochondria NOS to regulate mitochondrial O₂ uptake and H₂O₂ production through the interaction of NO with the respiratory chain has recently been studied [40].

3 Iron metabolism and oxidative stress

3.1 Body iron metabolism

3.1.1 Nutritional sources of iron

The maintenance of stable extracellular and intracellular iron concentrations requires the coordinated regulation of iron transport into plasma from dietary sources in the duodenum, from recycled senescent red cells in macrophages, and from storage in hepatocytes. In humans, the most widely used indices of Fe status are hemoglobin concentration, transferrin saturation, and serum ferritin. Iron deficiency (ID) and its associated anemia may contribute to reduced energetic efficiency, lower aerobic capacity, decreased endurance, and fatigue.

ID is the most common nutritional deficiency in the world. When iron intake fails to meet physiological or increased demands for iron, for example, owing to bleeding or inadequate absorption, iron stores become depleted and ID develops. Various types of ID exist in clinical conditions. Common classification differentiates between absolute iron deficiency, functional iron deficiency, and iron sequestration [41]. Oral iron therapy is often adequate to treat absolute iron deficiency and mild to moderate cases of ID. In cases of severe ID, anemia of chronic disease, and functional iron deficiency, intravenous (iv) iron therapy is often more effective. A number of oral and iv iron preparations are available. Oral iron preparations include iron(II) salts, polysaccharide iron(III) complexes, and

combinations of iron, for example, with folic acid or vitamin C [42].

There are three sources of iron in the diet: native food iron (either heme or nonheme iron), fortification iron (nonheme iron that has been intentionally added to food during manufacture), and contaminant iron (nonheme that is unintentionally incorporated into food as a result of either chemical reactions or mechanical processing during food preparation) [43]. A variety of nonheme and heme sources of iron are listed in Table 1. The chemical forms of iron in food influence the amount absorbed through the gastrointestinal tract and into the bloodstream. The relatively low bioavailability of iron from cereal grains and legumes is attributed to the phytic acid content of these foods. Significant data indicate that inhibitors of iron absorption include phytate, polyphenols, soy protein, and calcium, and enhancers include animal tissue and ascorbic acid [44]. Nutritional iron comes from two sources, heme and nonheme. Heme iron is obtained from meat sources and is more readily absorbed than nonheme iron obtained from the consumption of grains and vegetables. Heme is absorbed as the stable porphyrin complex that is unaffected by other food components. Heme is hydrophobic and thus has been proposed to diffuse passively through plasma membranes.

The absorption of heme iron is five to ten times greater than that of nonheme iron. Although heme represents only 10–15% of dietary iron in meat-eating populations, it may account for nearly 40% of total absorbed iron. Because the absorption of heme iron is constant and independent of meal composition, the contribution of heme iron can be readily calculated from dietary records [45].

Recently, however, an intestinal heme transporter was identified and named heme carrier protein 1. This transporter is abundantly expressed on the brush border of enterocytes in the duodenum [46]. Concerning nonheme iron, it exists in the form of “free” complexes. The absorption of iron is markedly influenced both by the iron status of the subject and a great number of dietary factors. The absorption of iron from the diet is thus determined more by meal composition than by the amount of iron present in the diet.

Eight enzymes, which are divided between the mitochondria and cytoplasm, are involved in the heme biosynthesis pathway. Heme is generated in the mitochondrial matrix. The first step in mammalian heme biosynthesis is catalyzed by the enzyme 5-aminolevulinic acid (ALA) synthase (ALAS). ALAS catalyzes the condensation of glycine with succinyl-CoA to form ALA and CO₂. The mature protein is located in the mitochondrial matrix. Once ALA is produced by ALAS, it is exported out of the mitochondrial matrix to reach the second pathway enzyme. The terminal step of heme synthesis is the insertion of ferrous iron into the protoporphyrin IX macrocycle to produce protoheme IX (heme) [47]. This is catalyzed by the enzyme ferrochelatase that is synthesized in the cytoplasm as a preprotein and is translocated to the mitochondrial matrix, where it is associated with the inner mitochondrial membrane [48].

3.1.2 Importance of heme oxygenases in enterocytes

Despite the clear importance of dietary heme as a source of body iron, the mechanism by which the enterocyte takes up heme and catabolizes it to utilize the iron is poorly understood. Heme is degraded inside internalized vesicles within 2–3 h of heme uptake by receptor-mediated endocytosis. The role of HO localized in enterocytes has now been demonstrated [49]. HO is an enzyme that catalyzes the mixed function oxidation of heme using cytochrome P-450; NADPH; and molecular oxygen producing CO, iron, and biliverdin [50]. Three isoforms of HO have been characterized: an inducible form (HO-1), which is upregulated especially in the spleen and liver in response to various types of stress, and two constitutive forms (HO-2 and HO-3). HO-1 and HO-2 are the products of distinct genes (ho-1, ho-2, also specified as hmox1, hmox2). HO-1 occurs at a high level of expression in the spleen and other tissues that degrade senescent red blood cells, including specialized reticuloendothelial cells of the liver and bone marrow, but is also present in enterocytes [51]. HO-2 is constitutively expressed in selected tissues (brain, liver, gut, and testis) and is involved in signaling and regulatory processes. HO-2 has three cysteine residues that have been proposed to modulate the affinity for heme, whereas HO-1 has none. It is likely that the cysteine residues in HO-2 are involved in some form of protein–protein interactions related to the signaling roles of the protein [52]. The existence of a third HO isoform, HO-3, was reported in the rat. The HO-3 transcript was found in a series of organs, including spleen, liver, kidney, and brain [53]. Clearly, it appears that the role of HO-1 and HO-2 in enterocytes needs to be investigated in pathological situations.

3.1.3 Physiopathological consequences of iron deficiency or accumulation

Iron is necessary for cells due to its ability to transport oxygen and electrons. Recently, the health effects of iron ex-

cess or deficiency have received increased attention as iron has been purported to play a role in the etiology of several chronic diseases, including diabetes, cardiovascular disease, and cancer. Iron has relevant biological functions, for example, the formation of hemoglobin, myoglobin, and numerous enzymatic groups. For a long time, the influence of ID was underestimated especially in terms of the worsening of cardiovascular diseases and the development of anemia. In contrast and paradoxically, excess iron accumulation causes organ dysfunction through the production of ROS. The iron redox couple mediates the transfer of single electrons through the reversible oxidation/reduction reactions of Fe²⁺ and Fe³⁺. Iron is an essential metal for hemoglobin synthesis in erythrocytes, oxidation–reduction reactions, and cellular proliferation [54].

The total amount of iron in a 70-kg adult is approximately 4 g, of which two thirds is the iron in red blood cells and 300 mg is in the myoglobin of muscles. The majority of this iron comes from the recycling of senescent erythrocytes by macrophages of the reticuloendothelial system (about 20 mg/day; Fig. 2). Most of the iron in plasma is directed to the bone marrow for erythropoiesis. More than 2 million new erythrocytes are produced every second by the bone marrow, requiring a daily supply of at least 20–30 mg of iron. Therefore, a majority of the iron comes from the recycling of senescent erythrocytes by macrophages of the reticuloendothelial system. Only 1–2 mg of the daily iron supply is derived from intestinal absorption, which, under a steady state, is sufficient only to replace the insensible iron loss. Significant amounts of iron are also present in macrophages (up to 600 mg), whereas excess body iron (~1 g) is stored in the liver. To ensure adequate iron content, mammalian cells regulate iron levels through uptake and export (Fig. 3). Macrophages export Fe²⁺ from their plasma membrane via ferroportin (FPN), in a process coupled by reoxidation of Fe²⁺ to Fe³⁺ by ceruloplasmin and followed by the loading of Fe³⁺ to transferrin (Tf). Tf is the major iron transporter protein in the body [55]. Exported iron is scavenged by Tf that maintains Fe³⁺ in a redox-inert state and delivers it into tissues. Iron enters the cell from the bloodstream in a complex with Tf, which binds to Tf receptor (TfR) on the plasma membrane, followed by receptor-mediated endocytosis [4, 56, 57]. Under physiological conditions, plasma Tf is hyposaturated (to approximately 30%) and displays a very high iron-binding capacity. TfR expression is controlled by a variety of cellular conditions, including iron and oxygen status.

The absorption of dietary iron, which is present in either a heme or nonheme form, is carried out by mature villus enterocytes of the duodenum and proximal jejunum. Much of the iron that enters the lumen of the duodenum in the diet is in the oxidized or ferric form and, therefore, must be reduced before it can be taken up by enterocytes [58]. The reduction of iron is probably carried out enzymatically by a brush border ferric reductase. Identification of the transporters and other proteins functioning in the absorption of dietary nonheme and heme iron has greatly enhanced the

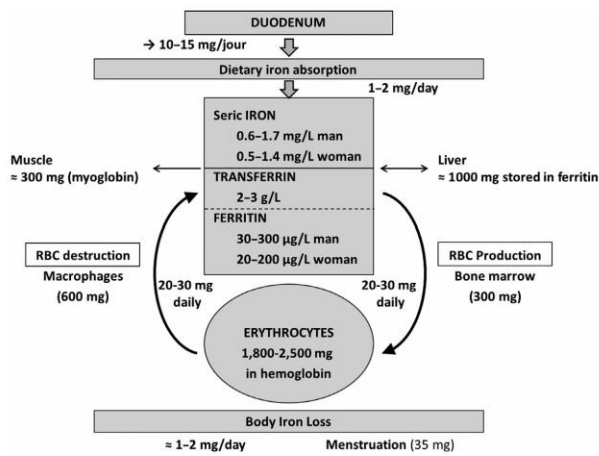


Figure 2. Iron distribution in the adult human body. A 70-kg adult has about 4 g of total body iron. Most of iron is circulating in the hemoglobin of the red blood cell (RBC). The body absorbs only 1–2 mg daily from a diet containing 10–15 mg of iron.

understanding of this process, which is central to the control of body iron content. Nonheme iron is absorbed through the action of divalent metal transporter 1 (DMT1) and duodenal cytochrome b, which are mammalian plasma ferric reduc-

tase enzymes that catalyze the reduction of ferric to ferrous ion in the process of iron absorption. These reductases are present at the apical membrane in the proximal small intestine. There are two isoforms of DMT1 protein that differ at

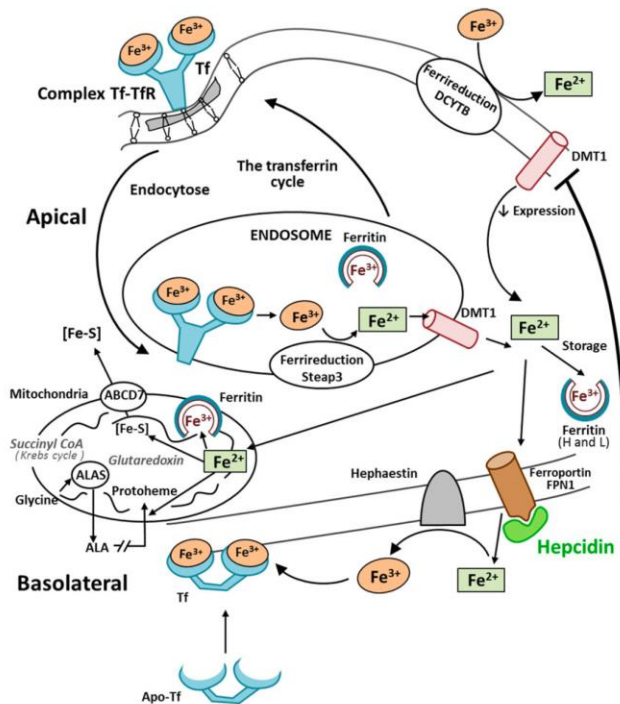


Figure 3. Control of iron homeostasis in the enterocyte. Fe^{3+} -Tf binds to TfR on the cell surface and undergoes receptor-mediated endocytosis. The released Fe^{3+} is reduced to Fe^{2+} by the ferrireductase 6-transmembrane epithelial antigen of the prostate 3 within the endosome before export from the endosome by a DMT1. Fe^{2+} that is not utilized or stored in ferritin is exported by FPN. Inside the mitochondrial matrix, Fe can be directed to different pathways, including iron-sulfur (Fe-S) cluster biogenesis. The clusters in the mitochondria are tightly regulated by a transporter: ATP-binding cassette transporter. The first step in mammalian heme biosynthesis is catalyzed by the enzyme ALAS. ALAS catalyzes the condensation of glycine with succinyl-CoA to form ALA and CO_2 . The terminal step of heme synthesis is the insertion of Fe^{2+} into the protoporphyrin to produce protoheme (heme).

their C-terminus. They have tissue-specific roles in iron absorption and exhibit different subcellular trafficking [59]. The amount of iron absorbed by enterocytes is influenced by a variety of factors, including variations in body iron stores, red cell levels, hypoxia, inflammation, and pregnancy [60].

The Tf–TfR complex is internalized to a unique endosomal compartment where acidification leads to the release of ferric iron. The ferric iron is then reduced to ferrous iron and transported across the endosomal membrane by the DMT1. The endosomal ferrireductase responsible for the reduction of ferric iron in erythroid cells was recently identified as the 6-transmembrane epithelial antigen of the prostate 3 (Steap3) protein [61].

Multiple physiological processes are involved in maintaining iron homeostasis. These include its storage at the intracellular and extracellular levels. The control of iron balance in the whole organism requires communication between sites of uptake, utilization, and storage [62]. Key protein transporters and the molecules that regulate their activities have recently been identified. Ferritins and hepcidin are the major regulator proteins.

Ferritins are composed of 24 subunits that fold in a 4-helical bundle to form an almost spherical protein shell. Ferritin can accumulate up to 4500 iron atoms as a ferrihydrite mineral in a protein shell and releases these iron atoms when there is an increase in the cell's need for bioavailable iron [63, 64] (Fig. 4A). There is a strong equilibrium between ferritin-bound iron (Fe^{3+}) and the labile iron pool in cells (Fe^{2+}), by which ferritin prevents the formation of ROS mediated by the Fenton reaction. Various cell types contain a transient cytosolic pool of iron, presumably bound to low molecular mass intracellular chelates, such as citrate, various peptides, ATP, AMP, or pyrophosphate. This labile iron pool is redox-active. The concentration of this labile iron pool is determined by the rates of iron uptake, utilization for incorporation into iron proteins (cytosol, mitochondria, and nucleus), storage in ferritin, and iron export from the cell.

Two functionally and genetically distinct ferritin subunits exist as follows: L-ferritin and H-ferritin (also known as light chain and heavy chain ferritin). Ferritin is found in the cytoplasm, nucleus, and mitochondria of cells. Serum ferritin has been thought to reflect iron stores in the body and to increase as a secreted byproduct of intracellular ferritin synthesis. Even though cellular iron is stored primarily in the cytoplasm, mitochondria use most of the metabolically active iron [65].

Because of its important function in the storage of iron, ferritin is ubiquitous in tissues, serum, and in multiple other locations within the cell. Iron is imported through endocytosis of Fe^{3+} -loaded Tf, which interacts with TfRs in a tightly regulated feedback loop. Iron regulatory proteins 1 and 2 (IRP1 and IRP2) record cytosolic iron concentrations and posttranscriptionally regulate the expression of iron metabolism genes. These actions are implicated in the optimization of cellular iron availability. IRP1 and IRP2 bind to iron-responsive elements located in regions of mRNAs that

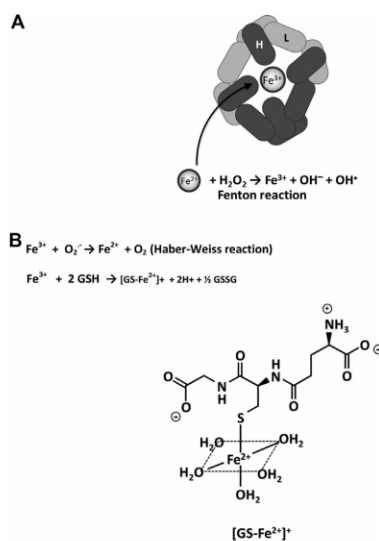


Figure 4. Ferritin and iron metabolism. (A) Ferritin is composed of 24 subunits that can be heavy (H) or light (L), with various ratios of H to L in different cell types and physiological conditions. Ferritin H has ferroxidase activity to convert Fe^{2+} to Fe^{3+} inside ferritin. Ferritin, when binding a $[4\text{Fe-4S}]$ cluster, is a functioning (cytoplasmic) aconitase. The majority of iron-sulfur (Fe-S) clusters are made in the mitochondria. (B) Fe^{3+} is rapidly reduced to Fe^{2+} in the presence of glutathione, the reducing of GSH being sufficient to reduce Fe^{3+} to Fe^{2+} at pH 7. GS- Fe^{2+} binds to glutaredoxins, proteins that are required for iron cluster assembly and heme biosynthesis.

encode proteins involved in iron uptake, storage, utilization, and export (Fig. 1) [62]. Iron metabolism is also transcriptionally regulated by the action of hypoxia-inducible factors (HIFs) that consist of a cytosolic protein (HIF-1 α) and a nuclear HIF-1 β subunit and form a DNA-binding heterodimer [66].

The recently discovered iron regulatory hormone, hepcidin, plays a critical role in controlling iron absorption through its ability to bind to and control the cell surface expression of FPN, FPN being a transmembrane protein that transports ferrous iron. Produced by the liver and secreted into the bloodstream, hepcidin regulates iron metabolism by inhibiting iron release from cells, including duodenal enterocytes. Human hepcidin is a 25 amino acid peptide. The structure of bioactive hepcidin is a simple hairpin with eight cysteines that form four disulfide bonds in a ladder-like configuration [67]. When iron stores are adequate or high, the liver produces hepcidin that circulates to the small intestine. The iron exporter FPN is required for iron transport from iron exporting cells. However, ferrous iron must be oxidized to ferric iron by ferroxidases before the iron can bind to Tf.

FPN is also the receptor for hepcidin [68]. Hepcidin binds to FPN1, which is present on the cell surface, and induces the phosphorylation of FPN, thus triggering the internalization of the hepcidin–FPN complex, leading to the ubiquitination and lysosomal degradation of both proteins. Hepcidin deficiency results in the development of systemic iron overload caused by excessive iron absorption. In the absence of hepcidin, FPN expression on the basolateral surface of enterocytes is increased, thus increasing the transport of dietary iron into the plasma. Copper-containing ferroxidase hephaestin assists by converting Fe^{2+} to Fe^{3+} that is then bound by Tf. Moreover, HIF-1 α and HIF-2 α have been shown to increase intestinal iron absorption, iron uptake into erythroid progenitors and heme synthesis, and to suppress hepcidin production thus ensuring an adequate supply of iron to support erythropoiesis [69,70]. Besides being produced by the liver, hepcidin is also believed to be produced by macrophages, fat cells, and the heart, but at a much lower level. The relevance of the extrahepatic production of hepcidin is still unclear, but it could play a role in the local regulation of iron fluxes [71].

3.2 Mitochondria and iron

Mitochondria represent a significant store of iron within cells, as iron is required for the functioning of respiratory chain protein complexes. The mechanism for intracellular iron trafficking to mitochondria is incompletely understood. It is accepted that cells use iron in mitochondria for the synthesis of heme and iron–sulfur (Fe–S) clusters (Fig. 3). The sequential flow of electrons in the respiratory chain, from a low reduction potential substrate to O_2 , is mediated by protein-bound redox cofactors. In mitochondria, heme, together with flavin, iron–sulfur, and copper cofactors mediates this multielectron flow [72]. The insertion of a ferrous iron atom into the porphyrin macrocycle by the enzyme ferrochelatase creates heme. It is now well demonstrated that mitochondria perform crucial roles in iron metabolism in that they synthesize heme, assemble iron–sulfur (Fe/S) proteins, and participate in cellular iron regulation [73]. Fe–S clusters are important cofactors of numerous proteins involved in electron transfer, and metabolic and regulatory processes [74].

The respiratory chain relies on iron-containing redox systems in the form of complexes I–III with Fe–S clusters and cytochromes with heme as prosthetic groups. As we previously reported, Complexes I and III are the main sites of $\text{O}_2^{\cdot-}$ generation. Complex I (NADH: ubiquinone oxidoreductase) oxidizes NADH from the tricarboxylic acid cycle and β -oxidation, and transports protons across the inner membrane. It is a major contributor to the cellular production of ROS. The redox reaction of complex I is catalyzed in the hydrophilic domain; it comprises NADH oxidation by a flavin mononucleotide. The energy transduction by complex I comprises NADH oxidation by a flavin mononucleotide, intramolecular electron transfer from the flavin to bound quinone along a chain of (Fe–S) clusters, quinone reduction, and proton translocation

[75]. Apart from complex I (NADH: ubiquinone oxidoreductase), the mitochondrial cytochrome bc1 complex (complex III; ubiquinol: cytochrome c oxidoreductase) has been identified as the main producer of superoxide and ROS within the mitochondrial respiratory chain. The formation and assembly of the respiratory chain enzymes is a complicated process that requires the assistance of a multitude of additional proteins and Fe–S clusters. The trafficking and storage of Fe–S clusters in the mitochondria is tightly regulated by a transporter: ATP-binding cassette transporter [76]. Furthermore, complex III generated ROS seem to act as second messengers in the context of cardioprotective ischemic and pharmacological preconditioning [77].

Studies have investigated changes in systemic iron homeostasis in animals and humans with cardiovascular diseases and characterized the intrinsic defects in iron regulatory pathways of failing cardiomyocytes. It has been demonstrated that the accumulation of iron in mitochondria can cause or exacerbate cardiomyopathy. This is the case in Friedreich's ataxia (FRDA), a human genetic disease caused by GAA triplet expansion in the FXN gene-causing frataxin deficiency [78]. Frataxin deficiency in FRDA causes mitochondrial iron dysmetabolism. Treatment of patients with a combination of the mitochondria-permeable iron chelator deferiprone and an antioxidant partially reversed the cardiac phenotype observed in FRDA, thus supporting the role of mitochondrial iron in the pathophysiology of cardiac dysfunction. In heart failure, it has been demonstrated that iron accumulated in the mitochondria. The activities of mitochondrial Fe–S cluster proteins were reduced in these hearts, and the defect in complex III activity was specifically attributed to the lack of an Fe–S cluster center [79]. On the basis of a number of biochemical arguments, it has been suggested that GSH plays a role in iron metabolism (Fig. 4B). However, it was concluded that GSH might not be directly involved or play an essential role in iron delivery to the mitochondrion [80]. Fe^{3+} is rapidly reduced to Fe^{2+} in the presence of GSH; the reducing potential of GSH being sufficient to reduce Fe^{3+} to Fe^{2+} at pH 7. In addition to an Fe^{2+} buffering role, Fe^{2+} -GS (glutathione synthase) binds to glutaredoxins, proteins that are required for iron cluster assembly and heme biosynthesis.

4 Oxidative stress and cardiomyocytes

4.1 Cardiomyocyte viability

During the early postnatal period, cardiomyocyte replication in the mammalian myocardium becomes undetectable. The number of cardiomyocytes of mature mammalian heart remains constant throughout life [81]. In adult hearts, mitotic division of cardiac myocytes appears undetectable as cardiomyocytes become terminally differentiated. The majority of mature cardiomyocytes are growth arrested at the G0 or G1 phase. The population of cardiomyocytes was thought to remain stable in number and with a one-to-one ratio to the

number of capillary microvessels providing oxygen and substrate delivery, not only during postnatal physiological growth of the heart, but also in the adult heart [82]. In these conditions, persistent stress leads to an ultrastructural remodeling in which cardiomyocyte death exceeds cardiomyocyte renewal, resulting in progressive heart failure. Recently, studies revealed that mammalian cardiomyocytes retain some capacity for division and identified endogenous cardiac progenitor cells in the heart; but the turnover rate of these cells is certainly limited. However, there is no consensus yet about the possibility of new cardiac myocyte generation [83].

Major sources of ROS are present in the myocardium [3], and paradoxically, a low level of antioxidant defenses exists in this tissue compared with other tissues such as liver. The activities of three enzymes (SODs, catalases, and GPx), which are able to detoxify activated oxygen, were determined in both the heart and liver. Cardiac muscle contains 150 times less catalase and nearly four times less SOD than does the liver. GPx activities were, however, similar in the two tissues [84, 85]. In myocardium, as in some other tissues, the reaction of radicals in the presence of O₂, or singlet oxygen, with some amino acids, peptides, and proteins yields hydroperoxides. Protein hydroperoxides are capable of initiating further radical chain reactions both intra- and intermolecularly, in particular in cardiomyocytes, thus inducing heart failure [86].

In cardiomyocytes, the mitochondria are located near calcium-release sites on the sarcoplasmic reticulum (SR) and can capture a large quantity of the released calcium. Accumulating evidence supports the critical role of biochemical cross-talk between the SR and mitochondria in normal cardiomyocyte viability and excitation contraction (EC) coupling. The SR and mitochondria have different individual functions, but the coordination between these two cellular organelles is essential to support synchronous cardiomyocyte contraction/relaxation and to facilitate adaptation to changing metabolic demands. Mitochondrial ROS production can modify EC coupling. A number of Ca²⁺ channels or transporters, and also myofilaments are sensitive to redox modifications [87]. Furthermore, mitochondrial ion channels, such as the inner mitochondrial anion channel, the permeability transition pore, or uncoupling proteins, are activated by ROS [88].

Another mechanism related to oxidative stress and concerning the myocardium is immunity. Although autoimmunity is a well-established pathogenetic mechanism in several endocrine, rheumatic, and neurological disorders, it has only recently gained more attention in cardiac diseases. Recent studies suggest that the heart possesses an intrinsic system that is intended to limit tissue injury. It is suggested that this intrinsic stress response is mediated, at least in part, by a family of pattern recognition receptors that belong to the innate immune system [89]. Depending on individual genetic predisposition, heart-directed autoimmune reactions are supposed to emerge as a consequence of cardiomyocyte injury induced by inflammation, ischemia, or exposure to cardiotoxic substances [90].

The discovery and characterization of the toll-like receptor (TLR) family has led to better understanding of the innate immune system and its function in the different organs, such as those of the cardiovascular system. Through these TLRs, cardiomyocytes respond to endogenous or exogenous signals that may influence the pathophysiological responses to dilated cardiomyopathy [91]. Moreover, heart failure of diverse origin is also now recognized to have an important immune component, with TLR signaling influencing the process of cardiac remodeling and prognosis. Therefore, the inhibition of TLR signaling may be of great therapeutic benefit in several forms of heart failure [92].

4.2 RNOS, cardiomyocytes, and reticulum stress

The ER is an organelle that plays an essential role in many cellular processes, such as the folding of secretory and membrane proteins, calcium homeostasis, and lipid biosynthesis. The ER supports the biosynthesis of approximately one third of all cellular proteins in eukaryotic cells. To achieve the proper folding of proteins, the lumen of the ER is a special environment [93]. Evidence of an intimate relationship between the ER and mitochondria has accumulated. These close contacts establish extensive physical links that allow the exchange of lipids and calcium and they coordinate pathways involved in cell life and death [94]. Various factors, such as oxidative stress and disturbances of calcium homeostasis, both of which interfere with ER function, lead to the accumulation of unfolded proteins. The resulting ER stress triggers the unfolded protein response [95]. Conditions that perturb cellular energy levels, the redox state or Ca²⁺ concentration reduce the protein folding capacity of the ER. Cells have evolved a series of adaptive and protective strategies. If unresolved, ER stress is lethal to cells via ER stress-induced apoptosis [96].

The important role of maintaining Ca²⁺ homeostasis within the cell is consequently dependent upon the ER and its many Ca²⁺-binding chaperones, including heat shock proteins (HSP) and calreticulin (CRT). CRT is integral to the quality control of protein folding and Ca²⁺ storage and release within the ER. CRT is a central Ca²⁺-buffering protein that regulates Ca²⁺ storage and release within the ER [97]. Cell-surface CRT exposure is an important process that targets the cell for immunogenic cell death (ICD) [98]. The translocation of CRT to the cell surface can be induced by anthracyclines. Furthermore, CRT regulates the activity of Ca²⁺-ATPase, providing dynamic control of ER Ca²⁺ homeostasis [99, 100].

5 Cellular signaling in the cardiovascular system

5.1 (NADPH) oxidases and free radicals

All NOX family members are transmembrane proteins that transport electrons across biological membranes to reduce

oxygen to superoxide. There are conserved structural properties of NOX enzymes that are common to all family members, but activation mechanisms and tissue distribution of the different members of the family are markedly different [101]. The physiological functions of NOX family enzymes include cellular signaling, regulation of gene expression, and cell differentiation. NOX enzymes contribute to a wide range of pathological processes, such as the regulation of immunomodulation and cellular proliferation [102].

As we reported, the generation of $O_2^{\cdot-}$ and NO may lead to the production of the harmful molecule $ONOO^-$. It may result in S-nitrosylation and tyrosine nitration of proteins with a concomitant change in their function. It is a potent trigger of oxidative protein, DNA damage, including DNA strand breakage and base modification. It activates the nuclear enzyme poly-ADP ribose polymerase, resulting in energy depletion and apoptosis/necrosis of cells. Peroxynitrite has been associated with the regulation of cell death [103, 104].

5.2 Oxidative stress, immunogenic regulation, and transcription factors

A variety of transcription factors may be activated as a result of oxidative stress, leading to the expression of different genes, including those for growth factors, inflammatory cytokines, chemokines, cell cycle regulatory molecules, and anti-inflammatory molecules. A new concept of ICD has recently been proposed. The immunogenic characteristics of this cell death mode are mediated mainly by damage-associated molecular patterns (DAMPs), most of which are recognized by pattern recognition receptors. Some DAMPs (e.g., CRT and adenosine triphosphate) are actively emitted by cells undergoing ICD, whereas others are emitted passively (e.g., high-mobility group box 1 protein) [105]. Some DAMPs, such as high-mobility group box 1 protein, can be inactivated by oxidation. The ability of anticancer drugs such as anthracyclines to induce ICD was shown to depend on the induction of ER stress. The combined action of ROS and ER stress activates danger signaling pathways that help to traffic DAMPs to the extracellular space. The immunogenicity of ICD was found to be diminished in the presence of antioxidants [106].

In order to prevent oxidative stress, the cell must respond to ROS by activating an antioxidant defense system. Antioxidant enzymes play a major role in reducing ROS levels. The redox regulation of transcription factors is thus significant in determining the gene expression profile and cellular response to oxidative stress. Increasing the cellular antioxidant capacity by upregulating antioxidant detoxification genes is critical in cellular adaptation to oxidative stress and protection from oxidative damage. The antioxidant genes are regulated by a highly homologous enhancer termed the antioxidant responsive element (ARE), or electrophile response element [107]. Bach 1, a transcription factor, forms a heterodimer with Maf (*Musca domestica* antifungal) oncoproteins and binds to the Maf-recognition element (MARE). Among the various tran-

scription factors, the activation of nuclear factor erythroid-related factor 2 (Nrf2) and nuclear factor kappa B (NF- κ B) is decisive for a wide range of processes associated with oxidative stress.

5.2.1 Nrf2

The Nrf2-Keap1 (Kelch-like erythroid cell derived protein with CNC homology (ECH) associated protein 1) signaling pathway (Nrf2-Keap1 system) is one of the most important cell defense and survival pathways. This system is a key factor for cell protection from oxidative and electrophilic insults that contribute to maintain the redox cellular microenvironment [108]. Nrf2 binding to ARE elements also triggers antioxidant programs that scavenge intracellular ROS [109]. Nrf2 is referred to as the “master regulator” of the antioxidant response, modulating the expression of hundreds of genes, including not only the familiar antioxidant enzymes, but large numbers of genes that control numerous processes, such as immune and inflammatory responses [110]. It is important to point out that the redox-dependent Nrf2 system plays a central role for HO-1 induction in response to oxidative stress. There are several critical regulatory domains present in a 10-kb region of the 5'-flanking sequence of the HO-1 gene. Two of the most highly studied enhancer regions, called E1 and E2, contain stress-response elements that structurally resemble the ARE. The major positive transcriptional regulator of the HO-1 gene that acts on StREs is Nrf2. Nrf2 is ubiquitinated by forming a complex with Keap1. Keap1 targets Nrf2 for ubiquitin-dependent degradation and, hence, represses Nrf2-dependent gene expression. Some HO-1 inducers of oxidative stress may prevent Keap1-dependent degradation of Nrf2, thereby resulting in the nuclear accumulation Nrf2. Accumulated or stabilized Nrf2 may bind to StRE regions to form complexes with the small Maf proteins, leading to the initiation of HO-1 gene transcription [111]. In response to nitrosative stress, the activation of Nrf2 signaling leads to the induction of HO-1 as a protective mechanism against inflammatory damage. LPS-induced inducible NOS (iNOS) expression was suppressed by pretreatment with HO-1 inducers in Nrf2^{+/+} macrophages [112].

5.2.2 NF- κ B

The activation of NF- κ B transcription factor is critical for a wide range of processes, such as immunity and inflammation, as well as cell development, growth, and survival. NF- κ B transcription factors consist of homo- or heterodimers of a group of five proteins, namely p65, RelA, RelB, c-Rel, NF- κ B1 (p50 and its precursor p105), and NF- κ B2. The NF- κ B pathway is activated by a variety of stimuli, including cytokines, ionizing radiation, and oxidative stress. Redox modulations of the NF- κ B pathway have been widely demonstrated. Oxidative stress can lead to chronic

inflammation by activating a variety of transcription factors, including NF- κ B, and inflammation itself has a reciprocal relationship with oxidative stress [113].

Under basal conditions, NF- κ B is contained in the cytoplasm by inhibitors of NF- κ B (I- κ B). In response to a wide range of signals, the regulatory NF- κ B subunits, p50 and p65, dissociate from I- κ B and subsequently translocate to the nucleus. In the absence of stimuli, NF- κ B is found in cytoplasm bound to the inhibitory I- κ B proteins (I- κ B α , I- κ B β , I- κ B γ , I- κ B ϵ , and Bcl3) [114]. In response to stimuli, including cytokines, mitogens, and oxidative stress, I- κ B proteins are rapidly phosphorylated by I- κ B kinase. Oxidative stress results in lipid peroxidation products or the depletion of reduced GSH and subsequent increases in cytosolic oxidized forms. GSSG, which is produced in response to oxidative stress, causes rapid ubiquitination and phosphorylation and thus subsequent degradation of I- κ B, which is a critical step for NF- κ B activation. By modulating a wide variety of transcription factors, cellular metabolism may be regulated.

In summary, Nrf2 modulates the anti-inflammatory cascade through the inhibition of NF- κ B, and regulates cellular antioxidant responses. Oxidative stress and inflammation are two components of the natural host response to injury and are important etiologic factors in atherogenesis. The Nrf2/ARE pathway has been identified as an endogenous atheroprotective system for antioxidant protection and the suppression of redox sensitive inflammatory genes [115].

6 Iron metabolism and cardiovascular diseases

Dysfunction in the failing myocardium may result from oxidative stress disequilibrium. Growing evidence suggests that modifications in oxidative stress may be induced by ionic metals, such as iron. In patients with heart failure consecutive to dilated cardiomyopathy, we demonstrated alterations in iron levels in different parts of the myocardium and that these were related to functional parameters. These findings revealed a relationship between iron metabolism disturbances and the severity of heart diseases [3].

6.1 Coronary heart diseases (CHDs)

Observational studies have indicated that higher exposure to iron may be associated with a higher risk of CHD. The epidemiologic evidence, however, is still ambiguous. Some studies have found no association or an inverse association between iron and CHD incidence or mortality. Recently, the characteristics of 32 separate cohorts from 21 studies were reported [116]. In this quantitative meta-analysis of prospective cohort studies, total iron intake was inversely associated with the incidence of CHD, whereas heme-iron intake was positively associated with incidence. Nonheme iron was not found to be associated with CHD incidence. In contrast, heme iron

increased the risk of CHD. An hypothesis of these results is in relation with the iron bioavailability, heme iron is absorbed at a much greater rate than is nonheme iron [117].

It has been proposed that a state of sustained iron depletion or mild ID exerted a primary protective action against CHD—the so called “iron hypothesis”; the protective effect of iron depletion, which may have multiple beneficial consequences, is due to decreased availability of redox-active iron *in vivo*. One persistent criticism of the iron hypothesis is that atherosclerosis may not be a prominent feature of hereditary hemochromatosis (HH). HH is a clinically and genetically heterogeneous late-onset autosomal recessive disorder that leads to excess iron accumulation in multiple tissues, including the heart. Epidemiological evidence of the possible effect of HFE gene variations on CHD is equivocal. HH can be caused by HFE mutations, and HFE gene variations were shown to be associated with CHD and atherosclerosis [118,119]. Recently, the goal of one study was to assess the contribution of four HFE gene variants to the risk of CHD. Some HFE gene mutations were shown to be associated with iron overload and CHD [120]. Interestingly, HFE mutations were associated with a 70% increased risk of CHD in women but not in men [121].

One of the most interesting aspects of CHD epidemiology is the gender difference. In all age groups, women have a lower risk of myocardial infarction (MI) than do men. Epidemiological observations and the results of recent controlled clinical trials suggest that it is not estrogen that protects women. An alternative hypothesis was suggested, namely that the lower risk of heart disease in women may be due to gender differences in iron metabolism. It has been suggested that lower body iron stores, and not the loss of ovarian function, explained the differences between men and women in the development of CHD [122].

6.2 MI

Studies indicate that high consumption of red and/or processed meat is related to an increased risk of cardiovascular death. These associations have been ascribed, at least partly, to the heme iron content in red meat [123]. Prospective studies have investigated the association between heme iron and nonheme iron intake and the risk of fatal and nonfatal AMI (where AMI is acute MI). The results did not support the hypothesis that dietary iron in general increases the risk of coronary disease. They suggested, however, that a high intake of heme iron was associated with an increased risk of MI [124,125]. A recent prospective study examined heme iron and nonheme iron intake in relation to the risk of fatal and nonfatal AMI separately. In this study, a positive association between heme iron intake and fatal AMI was particularly pronounced in men with low intakes of calcium, magnesium, and zinc. It was hypothesized that in the conditions of low mineral intake, the interactions between iron and mineral intake may lead to increased iron absorption [126].

6.3 Arrhythmia

Left ventricular and atrial function in patients with pathologies, such as HH or β -thalassemia, may be affected directly by iron deposition. The association between cardiac arrhythmia and HH has been well documented. Most of the published studies have described cases of cardiac arrhythmia in the presence of heart failure due to iron overload. The main clinical finding was progressive congestive heart failure (CHF) with arrhythmia, and autopsy confirmed excessive siderosis of the heart [127]. It is therefore difficult to determine whether the cardiac arrhythmia detected in subjects with HH is directly linked to systemic iron overload rather than to heart failure. Recently, a study reported that the incidence of both supraventricular and ventricular ectopy tended to be greater in the combined HH groups than in age- and gender-matched volunteer control subjects [128].

In thalassemia, electrocardiogram abnormalities are common. Heart rate modifications and prolonged corrected QT interval (QTc) were present, regardless of cardiac iron status. Repolarization abnormalities, including QT/QTc prolongation, left shift of the T-wave axis, and interpretation of ST/T-wave morphology, were the strongest predictors of cardiac iron [129]. Concerning the incidence of iron overload on atrial functions, studies have demonstrated that atrial volume and function are decreased in these conditions. In subjects without detectable cardiac iron, the left atrial ejection fraction declined with age [130]. Patients suffering from iron-overload cardiomyopathy have impaired systolic and diastolic function, and increased susceptibility to atrial fibrillation (AF). The development of AF remains poorly understood and is likely to vary significantly among different AF pathologies. AF is classified as paroxysmal (paroxysmal AF, PAF), persistent, or permanent AF depending on the timing and duration of episodes. In all these pathologies, studies have documented the long-term benefits of iron-chelation therapy on electrocardiographic abnormalities [131, 132].

6.4 Anemia in patients with heart disease

Approximately one third of patients with CHF and 10–20% of those with CHD also have anemia [133]. The treatment of anemia in patients with CHF and reduced left ventricular ejection fraction has traditionally focused on erythropoietin-stimulating agents (ESAs) [59]. Recently, large randomized controlled trials of ESA and iv iron supplementation therapy have been completed. ESA therapy did not show any improvement in clinical outcomes, but iv iron supplementation may be of benefit in improving HF symptoms [134]. In patients with CHF, erythropoietin (EPO) is highly expressed, and a close correlation between EPO and CHF has been demonstrated [135]. The correction of anemia with EPO has reduced morbidity and mortality in patients with chronic kidney disease, and recent studies have shown that the same approach was not always beneficial in CHF patients. The use of EPO in

patients with cardio-renal-anemia syndrome was associated with an increased risk of death, along with a trend toward an increased risk of CV events and stroke [136].

6.5 Atherosclerosis

The mechanism whereby iron may stimulate atherogenesis has been intensively investigated. Many excellent reviews on current knowledge with regard to atherosclerosis are available. Atherogenesis is characterized by inflammatory changes, leading to plaque formation and then to plaque rupture and arterial thrombosis. Atherosclerotic lesions are preceded by fatty streaks, accumulations of lipid-burdened cells, below the endothelium. Most of the cells in these fatty streaks are macrophages, together with T cells. The transendothelial migration of leukocytes is a fundamental inflammatory mechanism in atherogenesis. This process is in part mediated by the interaction between endothelial adhesion molecules and their ligands on monocytes [137]. Epidemiological studies and experimental data suggest that iron is involved in the onset of atherosclerosis. The relationship between iron and atherosclerosis is complex and remains contradictory. Oxidative stress has been implicated in the etiology of atherosclerosis, and transition metals such as iron aggravate oxidative stress. Iron plays an important role in the pathogenesis of atherosclerosis, primarily by acting as a catalyst for the atherogenic modification of LDL. Furthermore, macrophages play a key role in atherogenesis through their proinflammatory action, which involves the production of IL-1 β and tumor necrosis factor. In our laboratory, we demonstrated that in rabbits, hypercholesterolemia induced increased levels of superoxide iron deposition in the aorta. Our experimental model focused on the very early stage of atherogenesis when macrophages adhere to the endothelial layer [138].

Proinflammatory cytokines induce iron uptake by monocytes and macrophages. Iron promotes monocyte recruitment at the endothelium by modulating the expression of cell adhesion molecules on the endothelium. Iron chelators prevent these iron-mediated events [139].

Experimental evidence, relating to molecular chemistry, supports the oxidative hypothesis of atherosclerosis: excess iron generates oxidative stress and contributes to the pathogenesis of the disease. In clinical trials, the evidence concerning the specific role of iron in atherogenesis is often contradictory [140]. In patients with atherosclerotic lesions, iron has been detected in coronary plaques. In biopsied coronary arteries of patients with ischemic cardiomyopathy and atherosclerosis, we observed that iron content was high and associated with an increase in lipid peroxidation [1]. A majority of data are consistent with the hypothesis that high iron levels may contribute to atherosclerosis as one factor in a multifactorial disease. However, some epidemiological data suggest that high-stored iron levels are an independent risk factor for coronary artery disease [141]. In addition, iron-chelation therapy using desferrioxamine has been shown to slow down

the early stages of atherosclerosis, probably by decreasing iron levels [142]. Patients with coronary artery disease had impaired endothelium-dependent vasodilation in response to methacholine compared with healthy control subjects, and deferoxamine improved the blood flow response to methacholine in patients with coronary artery disease. Iron chelation thus appeared to improve NO-mediated, endothelium-dependent vasodilation in these patients [143].

7 Conclusions

If oxidative events are the basis for a number of pathologies, the evident therapy for all of them should be the use of antioxidants. However, no clear benefit of antioxidant therapy has yet been demonstrated in clinical intervention studies [144]. In contrast, it appears that iron plays a central role in the formation and the scavenging of ROS and RNS. It is therefore important to understand the mechanisms associated with the development of dysfunctional iron homeostasis. It is of pivotal importance to understand the precise mechanisms of how different iron compartments may be implicated in local oxidative stress. Iron-chelation therapy may present a novel way to interrupt the cycle of catalytic iron-induced oxidative stress and tissue injury [145, 146]. Iron chelators may be of therapeutic benefit in many pathological conditions. Advances have been made in major preclinical and clinical trials on iron-chelation therapy for the treatment of iron-overload disease as well as cardiovascular and chronic inflammatory diseases.

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3. Implications du fer, du stress oxydant et du risque cardio-métabolique préexistant dans la cardiotoxicité des anthracyclines

a) Place du stress oxydant et du fer dans la cardiotoxicité des anthracyclines

Bien que les mécanismes mis en jeu dans la cardiotoxicité des anthracyclines ne soient pas totalement élucidés, il est maintenant admis qu'ils reposent essentiellement sur une majoration d'un processus oxydatif mettant en jeu la production d'espèces radicalaires (19, 24). La présence, dans l'organisme, d'une production contrôlée de radicaux libres de l'oxygène est indispensable (86). Ils participent aux voies de signalisation impliquées dans la croissance, la protection, la différenciation et la mort cellulaire. Cependant, une surproduction de ces espèces radicalaires joue une composante dans l'étiologie du vieillissement, dans les phénomènes ischémiques et tumoraux ainsi que dans les désordres immunitaires et endocriniens. L'implication du stress oxydant dans la cardiotoxicité des anthracyclines a été confirmée par l'étude de la 5-iminodaunorubicine. Cette molécule, non commercialisée du fait de sa myélotoxicité, fait partie de la famille des anthracyclines mais possède une structure chimique différente des autres composés de cette classe. En effet, ses fractions quinones sont remplacées par des groupements imines. Il en résulte une réduction de l'ampleur des lésions myocardiques qui serait liée à une diminution de la production d'espèces oxydantes (87). Du fait de l'extrême réactivité des anthracyclines, différentes voies participent à la production d'espèces radicalaires par ces agents pharmacologiques. A l'exception de la 5-iminodaunorubicine, les anthracyclines possèdent une structure de type quinone qui leur permet de fonctionner comme accepteurs et donneurs d'électrons. Initialement, les anthracyclines étaient considérées pour leurs propriétés antibiotiques dérivant d'un bacille : l'*Actinomycete Streptomyces* (45). Elles possèdent une partie chromophore composée de 4 anneaux aromatiques et sont constituées de fractions quinones et hydroquinones. Les fractions quinones peuvent être réduites en semi-quinones sous l'action des NADPH oxydases (88). Ainsi, leur réduction en semi-quinones radicalaires conduit à la libération d'électrons libres et par là même à la formation de radicaux libres de l'oxygène tels que l'anion superoxyde $O_2^{\cdot-}$ et de l'hydroxyle $\cdot OH$ (89). Ces composés semi-quinones peuvent ensuite être recyclés en quinones par réduction de l'oxygène.

La première cible des radicaux libres générés par les anthracyclines est la mitochondrie (90). C'est également dans la mitochondrie que sont produites une grande partie des espèces radicalaires (23). La production mitochondriale d'espèces radicalaires est confirmée par les effets cardioprotecteurs de l'administration d'un antioxydant agissant au niveau de la mitochondrie : le Mito-Q. De plus, le traitement par la doxorubicine est associé à une surcharge en fer au sein de la mitochondrie *in vitro* comme *in vivo*, ce qui pourrait potentialiser la formation d'espèces radicalaires dans cet organelle et donc amplifier la cardiotoxicité de la chimiothérapie (91).

D'autres mécanismes interviennent dans la production radicalaire par les anthracyclines. Le premier fait intervenir le fer. Sous l'action des anthracyclines, l'ion ferrique Fe^{3+} se détache de ses sites de stockage et vient s'associer à trois molécules d'anthracyclines (Figure 3).

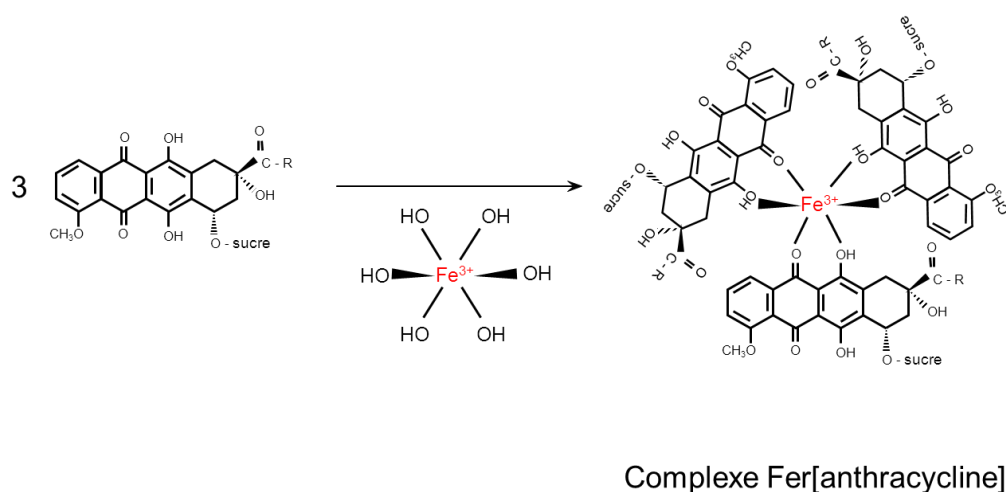


Figure 4. Formation des complexes Fer-anthracyclines.

Le complexe ainsi formé peut subir des réactions internes d'oxydoréduction et amplifier la production de radicaux libres de l'oxygène. Par ailleurs, les molécules d'anthracyclines peuvent s'associer au fer (92) pour former des complexes (Figure 3) générant des radicaux libres oxygénés (Figure 4) (93). Ce phénomène est auto-entretenu car le stress oxydant induit l'activation de protéines de régulation du métabolisme du fer, les IRP (Iron-Regulatory Protein) qui interagissent avec des récepteurs IRE (Iron Responsive Element) situés sur l'ARN messager du récepteur de la transferrine. Il en résulte une augmentation de l'expression du récepteur de la transferrine et une accumulation d'ions ferriques dans la cellule qui peuvent à nouveau fixer trois molécules d'anthracycline.

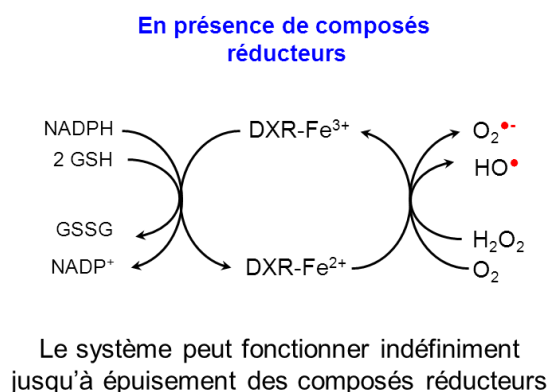


Figure 5. Formation de radicaux libres oxygénés par les complexes fer-anthracyclines.

Par ailleurs, il a été montré expérimentalement (87) que les fractions hydroquinones des anthracyclines pouvaient subir des peroxydations à l'origine de la formation de quinones électrophiles très réactives (Figure 1).

Cette surproduction de radicaux libres induit au niveau de toutes les structures cellulaires une augmentation de la peroxydation lipidique aboutissant à des altérations membranaires. Ces anomalies de la perméabilité membranaire ont de multiples conséquences sur l'homéostasie ionique. Les perturbations du flux calcique qui en découlent altèrent la contractilité myocardique. En plus des modifications architecturales et fonctionnelles des membranes cellulaires, le stress oxydant participe également à un remodelage de la matrice extracellulaire (88).

Les lésions mitochondriales induites par le stress oxydant participent à l'initiation de processus apoptotiques. Les radicaux libres déclenchent la libération, à partir de la mitochondrie, du cytochrome *c* qui active les caspases (6). Cette activation joue un rôle majeur dans les voies de signalisation de l'apoptose cellulaire. Par ailleurs, la majoration du stress oxydant participe à l'activation de différentes voies de kinases comme les MAP kinases (mitogen-activated protein kinase) ou les SAP kinases (stress-activated protein kinase) qui ont toutes les deux un rôle dans l'apoptose cellulaire. Le processus oxydatif se poursuit bien après l'arrêt du traitement par anthracyclines, expliquant l'apparition retardée de la cardiomyopathie dilatée induite par ces molécules.

On assiste par ailleurs, sous l'action des anthracyclines, à une augmentation de l'expression de la NO synthase inductible (iNOS) ainsi que de son ARN messenger au niveau du myocarde (10, 94). Le NO diffuse facilement au travers des membranes et des différents compartiments cellulaires ce qui permet son interaction avec l'anion superoxyde. La réaction entre l'anion

superoxyde et le NO, produit en grande quantité, conduit à la formation de peroxynitrites (ONOO⁻) au niveau des différentes structures cellulaires. Ces peroxynitrites produisent des dommages cellulaires qui potentialisent les altérations initialement induites par les formes radicalaires oxygénées : peroxydation lipidique, inactivation d'enzymes, inactivation de certaines protéines intervenant dans la contractilité cardiaque ou dans la chaîne respiratoire mitochondriale.

Le cœur est particulièrement vulnérable aux atteintes médiées par les espèces réactives de l'oxygène et au fer, du fait d'une faible expression des enzymes anti-oxydantes et de son métabolisme oxydatif important. Les protections anti-radicalaires peuvent être vitaminiques (vitamine, A, C, E, acide folique) et protéiques (enzymes de détoxification : superoxyde dismutases (SODs), catalases, glutathion peroxydases (GPX)) (95). En plus de la surproduction de radicaux libres, l'équilibre entre les espèces oxydantes et les « espèces » anti-oxydantes est perturbé par une déplétion des enzymes anti-oxydantes induite par les anthracyclines. Ainsi, sous l'action des anthracyclines, on assiste à une diminution des teneurs tissulaires de SODs et de GPX au niveau du tissu cardiaque (96). Cette diminution des défenses radicalaires serait impliquée dans des altérations de l'ADN mitochondrial et à des perturbations de l'efficacité de la chaîne respiratoire mitochondriale. Ces derniers persistent longtemps après l'arrêt du traitement par anthracyclines et pourraient expliquer le caractère retardé de la cardiotoxicité (97).

Outre l'action des anthracyclines, le statut oxydant et antioxydant des patients porteurs d'une néoplasie est modifié (98, 99). Les altérations de ce statut sont retrouvées aussi bien au niveau du tissu tumoral qu'au niveau plasmatique. Il est maintenant admis qu'au cours de l'évolution d'un cancer, situation associée à l'installation d'un état inflammatoire chronique, on assiste à une augmentation de la production de radicaux libres de l'oxygène, à une augmentation de la production de certaines espèces anti-oxydantes dans le but de « contrecarrer » cette majoration du stress oxydant et, à l'inverse, une diminution du taux d'autres substances anti-oxydantes par consommation, défaut d'apport ou défaut de production. Ce déséquilibre entre les substances oxydantes et anti-oxydantes au bénéfice des radicaux libres, participe à l'initiation et la promotion du processus tumoral ainsi qu'à sa diffusion métastatique. On retrouve, par exemple, une diminution du taux circulant d'acide ascorbique au cours du cancer du sein qui entraîne une diminution de la synthèse de collagène et donc du tissu conjonctif (99). Il en résulte une perte de l'intégrité de la matrice extracellulaire qui pourrait participer à l'initiation et au développement de la croissance tumorale.

b) **Incidences du risque cardio-métabolique préexistant sur la cardiotoxicité des anthracyclines**

Chez l'humain, la présence de facteurs de risque cardiovasculaires, et plus encore d'une cardiopathie préexistante, est un facteur déterminant dans l'apparition de la cardiotoxicité des anthracyclines. L'âge est l'un des principaux facteurs de risque du cancer du sein, mais aussi de la cardiopathie aux anthracyclines. Dans une méta-analyse portant sur plus de 4 000 patients, il a été démontré qu'il existait une relation forte entre l'âge et le développement d'une insuffisance cardiaque congestive après traitement aux anthracyclines (33). Ces résultats ont été confirmés par une méta-analyse plus récente, montrant qu'un âge >65 ans était associé à un risque 2,25 fois supérieur de cardiopathie aux anthracyclines, après ajustement aux autres facteurs de risque de cette cardiopathie (100). Il a également été démontré que la présence de facteurs de risque cardiovasculaires tels que l'hypertension artérielle, le diabète ou la dyslipidémie était associée à une plus grande incidence de cardiopathie aux anthracyclines (101).

Ce phénomène pourrait être expliqué par une moindre capacité adaptative des myocytes et de l'ensemble des mécanismes de défense, notamment radicalaires, à l'agression générée par les anthracyclines. En conséquence, à partir d'un certain niveau de risque cardiovasculaire, le traitement par anthracycline n'est pas proposé, ou différé. De nombreuses tentatives de prévention de l'apparition de la cardiopathie aux anthracyclines ont été proposées chez ces patients à haut risque, incluant un large panel médicamenteux (inhibiteurs de l'enzyme de conversion, bêta-bloquants, statines, anti aldostérone, ranolazine) et nutritionnels (omega-3, probucol) (102). Cependant, alors que ces molécules ont démontré une efficacité certaine dans des modèles précliniques cellulaires et animaux, la transposition chez l'homme n'a pas toujours été couronnée de succès. Une hypothèse pouvant expliquer les difficultés rencontrées est l'utilisation de modèles animaux non adaptés à la pathologie étudiée. En effet, les souris ou rats traités par ces molécules à visée cardioprotectrice sont le plus souvent indemnes de tout risque ou pathologie cardiovasculaire.

De plus, certaines études suggèrent que la présence d'une surcharge pondérale, d'une obésité ou un syndrome métabolique pourrait constituer un facteur de risque majeur de la survenue d'une cardiotoxicité aux anthracyclines. L'obésité est associée à un pronostic défavorable chez les patientes traitées par anthracyclines pour un cancer du sein (103, 104). A l'heure actuelle, il n'existe pas d'explication physiopathologique à l'augmentation de la sensibilité aux toxicités des anthracyclines chez les patients obèses. La dérégulation des adipokines,

hormones produites par les adipocytes, est impliquée le développement de multiples pathologies liées à l'obésité (105). Les taux plasmatiques d'adiponectine sont abaissés chez les sujets obèses. L'adiponectine protège de la cardiotoxicité des anthracyclines grâce à l'activation de la signalisation Akt dans les cardiomyocytes. Les souris knock-out (KO) pour l'adiponectine développent une dysfonction systolique plus marquée que des souris témoins après traitement par anthracyclines (106). L'apport d'adiponectine à ces souris KO permet de restaurer un niveau de cardiotoxicité identique à celui des souris témoins. Il a également été démontré que la metformine protège les cardiomyocytes de la toxicité des anthracyclines, et que l'adiponectine joue un rôle important dans cette action protectrice (107). De nombreuses études ont démontré que les cellules cancéreuses sont capables de reprogrammer les adipocytes. Ces adipocytes reprogrammés produisent des cytokines favorisant la croissance cellulaire et fournissant des lipides et d'autres métabolites aux cellules cancéreuses, le tout favorisant la croissance tumorale. Les anthracyclines altèrent le métabolisme glucidique et lipidique, induisant une augmentation des taux sanguins de cholestérol, triglycérides et HDL. Les anthracyclines diminuent l'expression du récepteur PPAR γ , ce qui empêche la prise de poids par inhibition de l'adipogénèse (108). Les interactions entre le tissu adipeux et les effets cardio-oxydatifs des anthracyclines pourraient donc constituer une piste thérapeutique prometteuse. Une étude chez le rat a démontré qu'un syndrome métabolique induit par un régime type « high-fat diet » était associé à une majoration de la toxicité cardiaque de la doxorubicine (109). Chez l'humain, un poids >70 kg est un facteur prédictif de cardiotoxicité chez des patientes recevant de la doxorubicine pour un cancer du sein métastatique (110). De plus, un indice de masse corporelle >27 kg/m² est associé à un risque de dysfonction ventriculaire gauche deux fois plus important (1,8% vs 0,9%) dans une population de patientes traitées par épirubicine pour un cancer du sein (111). Une des hypothèses évoquées pour expliquer ces résultats est que les patients obèses, en plus de cumuler plusieurs autres facteurs de risque cardiovasculaire, pourraient recevoir des doses plus importantes d'anthracyclines que les patientes de poids normal, car la dose de chimiothérapie est adaptée au poids ou à la surface corporelle (101).

Il est donc apparu intéressant dans ce cadre de développer et d'étudier un modèle de souris à haut risque cardiovasculaire, reproduisant ainsi la présentation clinique rencontrée en cardio-oncologie.

Etudes expérimentales

A. Paradoxically, iron overload does not potentiate doxorubicin-induced cardiotoxicity in vitro in cardiomyocytes and in vivo in mice

Ce travail a fait l'objet d'une publication parue en 2015 dans *Toxicology and Applied Pharmacology* dont on trouvera l'intégralité dans les pages à venir.

Objectifs

Dans un modèle cellulaire et murin de cardiotoxicité modérée à la doxorubicine, nous nous sommes proposés de déterminer l'impact d'une surcharge chronique en fer sur les incidences cardiotoxiques et oxydatives d'un traitement par cette chimiothérapie.

Résultats

Les principaux résultats sont les suivants :

- *In vitro*, nous avons démontré que dans les cellules cancéreuses EMT-6, une surcharge en fer n'altérerait pas les capacités antiprolifératives de la doxorubicine. En revanche, dans des cellules de type cardiomyocytes H9c2, un prétraitement par du fer modérerait la toxicité de celle-ci.
- *In vivo*, chez la souris C57/Bl6, nous avons réalisé une surcharge en fer préalable à un traitement par anthracyclines durant trois semaines. Nous avons pu mettre en évidence grâce à l'utilisation d'échocardiographie du petit animal, mais aussi d'expression génique (qPCR) de différents marqueurs de souffrance myocardique et de stress oxydatif (ainsi que par RPE) que la surcharge en fer (validée par dosages tissulaires de fer par spectroscopie atomique) semblait paradoxalement protéger le myocarde de la cardiotoxicité des anthracyclines. De plus, le traitement par doxorubicine modifiait considérablement la répartition tissulaire du fer, diminuant sa concentration cardiaque et augmentant sa concentration plasmatique.

Conclusion

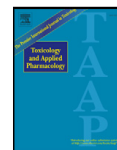
Une surcharge en fer, préalable à l'injection de doxorubicine, ne majore pas la cardiotoxicité de cette chimiothérapie. Au contraire, la mise en jeu des défenses anti-radicalaires suite à la surcharge en fer pourrait limiter les lésions oxydatives cardiaques générées par la doxorubicine. A la vue de ces données, le rôle des chélateurs du fer dans la cardioprotection contre les anthracyclines, déjà controversé, est remis en question. A la lumière de ces résultats et des données récentes de la littérature, nous discuterons dans la conclusion de ce travail des orientations possibles des recherches dans ce domaine.



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journal homepage: www.elsevier.com/locate/ytaapParadoxically, iron overload does not potentiate doxorubicin-induced cardiotoxicity *in vitro* in cardiomyocytes and *in vivo* in miceCharles Guenancia^{a,b}, Na Li^a, Olivier Hachet^{a,b}, Eve Rigal^a, Yves Cottin^{a,b}, Patrick Dutartre^a, Luc Rochette^a, Catherine Vergely^{a,*}^a INSERM UMR866, University of Burgundy, LPPCM, Faculties of Medicine and Pharmacy, Dijon, France^b Cardiology Department, University Hospital, Dijon, France

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ABSTRACT

Doxorubicin (DOX) is known to induce serious cardiotoxicity, which is believed to be mediated by oxidative stress and complex interactions with iron. However, the relationship between iron and DOX-induced cardiotoxicity remains controversial and the role of iron chelation therapy to prevent cardiotoxicity is called into question.

Firstly, we evaluated *in vitro* the effects of DOX in combination with dextran-iron on cell viability in cultured H9c2 cardiomyocytes and EMT-6 cancer cells. Secondly, we used an *in vivo* murine model of iron overloading (IO) in which male C57BL/6 mice received a daily intra-peritoneal injection of dextran-iron (15 mg/kg) for 3 weeks (D0–D20) and then (D21) a single sub-lethal intra-peritoneal injection of 6 mg/kg of DOX.

While DOX significantly decreased cell viability in EMT-6 and H9c2, pretreatment with dextran-iron (125–1000 µg/mL) in combination with DOX, paradoxically limited cytotoxicity in H9c2 and increased it in EMT-6. In mice, IO alone resulted in cardiac hypertrophy (+22%) and up-regulation of brain natriuretic peptide and β-myosin heavy-chain (β-MHC) expression, as well as an increase in cardiac nitro-oxidative stress revealed by electron spin resonance spectroscopy. In DOX-treated mice, there was a significant decrease in left-ventricular ejection fraction (LVEF) and an up-regulation of cardiac β-MHC and atrial natriuretic peptide (ANP) expression. However, prior IO did not exacerbate the DOX-induced fall in LVEF and there was no increase in ANP expression. IO did not impair the capacity of DOX to decrease cancer cell viability and could even prevent some aspects of DOX cardiotoxicity in cardiomyocytes and in mice.

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Introduction

Doxorubicin (DOX), an anthracycline antibiotic, is a broad-spectrum anticancer drug, particularly useful in the treatment of malignant lymphomas, acute leukemia, sarcomas and solid tumors such as breast, lung and ovary cancers (Young et al., 1981). However, despite good therapeutic results, the clinical use of DOX in chemotherapy is limited because it causes acute, sub-acute, and chronic cardiotoxicity (Horan

et al., 2006; Torti et al., 1986) leading to dose-dependent congestive heart failure, which may occur several years after treatment cessation (Steinherz et al., 1991), and may affect up to 20% of patients (Chatterjee et al., 2010; Singal and Iiskovic, 1998).

Several putative molecular mechanisms have been proposed to explain the cardiotoxicity of anthracyclines, but while the exact mechanism is still a matter of debate, it seems to be distinct from its anti-tumor activity since cardiomyocytes are minimally replicating cells (Shi et al., 2011). Oxidative stress is believed to be an important pathway in the cardiac side-effects of anthracycline therapy (Delemasure et al., 2007; Ghibu et al., 2012; Richard et al., 2011, 2008) and is related to the production of reactive oxygen and nitrogen species (RONS) during the intracellular metabolism of the quinone (Myers et al., 1977). Additionally, DOX forms stable complexes with ferric iron (Myers et al., 1982), and the iron in the complex undergoes reduction to ferrous iron, resulting in the generation of a semiquinone free radical of DOX (Gutteridge, 1984). The semiquinone then reacts with oxygen to form superoxide anion radical ($O_2^{\cdot-}$), which is converted into hydroxyl radical in the presence of iron through the Haber–Weiss reaction or forms peroxynitrite ($ONOO^-$) in the presence of nitric oxide ($^{\cdot}NO$) (Malisz

Abbreviations: ANP, atrial natriuretic peptide; AU, arbitrary unit; β-MHC, beta-myosin heavy chain; BNP, brain natriuretic peptide; cDNA, complementary deoxyribonucleic acid; DOX, doxorubicin; ECG, electrocardiogram; ESR, electron spin resonance; ET, ventricular ejection time; iNOS, inducible nitric oxide synthase; IO, iron overload; i.p., intra peritoneal; IR, ischemia/reperfusion; IVCT, isovolumic contraction time; IVRT, isovolumic relaxation time; IVS, inter ventricular septum; LVEDD, left ventricular end-diastolic diameter; LVEF, left ventricular ejection fraction; LVESD, left ventricular end-systolic diameter; LVMM, left ventricular mass normalized to body weight; LVPW, left ventricular posterior wall; mRNA, messenger ribonucleic acid; RONS, reactive oxygen and nitrogen species; RT-PCR, Reverse transcription polymerase chain reaction; SEM, standard error of the mean.

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and Hasinoff, 1995). In turn, these potent RONS induce lipid peroxidation and DNA damage (Muindi et al., 1984; Myers et al., 1982), thus promoting the death of cardiac cells by apoptosis or necrosis. Moreover, DOX-mediated RONS production may lead to the accumulation of iron in ferritin, and the DOX-iron complex impairs the DNA-binding activity of iron regulatory protein (IRP), leading to deregulation of iron homeostasis in cells (Gudjoncik et al., 2014). This process may lead to additional deleterious effects because iron is essential in several metabolic processes (Ammar et al., 2011; Xu et al., 2005).

Although the involvement of iron in anthracycline-induced cardiotoxicity is suggested by studies in which iron chelators were shown to be cardioprotective (Dorr, 1996; Poullart, 2004), some clinical trials led to disappointing results (van Dalen et al., 2008; Wouters et al., 2005). For now, the only iron chelator approved for the prevention of DOX-induced cardiotoxicity is dexrazoxane. However, this drug has been proved to prevent cardiotoxicity through several pathways (inhibition of topoisomerase II β , intracellular iron chelation, induction of hypoxia-inducible transcriptional factors). These multiple effects may explain why dexrazoxane is a more potent prophylactic agent than other iron chelators (Kalam and Marwick, 2013). In experimental models, iron loading was found to potentiate anthracycline cardiotoxicity in cardiomyocytes (Hershko et al., 1993; Link et al., 1996) and *in vivo* (Panjath et al., 2007) as well as in hemochromatosis gene *Hfe*^{−/−} mice (Miranda et al., 2003). However, the pharmacological interaction between iron and anthracyclines remains controversial and some authors found that DOX may paradoxically protect cardiomyocytes against iron-mediated cardiotoxicity (Corna et al., 2004), and that cardiomyocytes display a dose-dependent biphasic response to iron overload (Munoz et al., 2010).

The aim of our study was first to investigate *in vitro* the ability of dextran-iron to modify the DOX-induced deterioration of cell viability in cultured cancer cells (EMT-6) and in cardiomyocytes (H9c2). Secondly, we sought to investigate *in vivo* in mice, the impact of chronic tissue iron loading on DOX-induced nitro-oxidative stress and cardiotoxicity. We chose 20 days of daily 15 mg/kg dextran-iron loading since this iron formulation and dosing protocol was found to induce serum, hepatic and cardiac iron loading in C57Bl/6 mice (Moon et al., 2011; Wouters et al., 2005). We then deliberately chose a non-lethal dose of 6 mg/kg of DOX in order to reveal any potential deleterious effects of iron loading, and to be able to follow the evolution of myocardial function ten days after its injection. We evaluated iron status, nitro-oxidative stress levels and cardiac functional alterations. The major finding of our study was that, paradoxically, iron loading did not potentiate DOX cardiotoxicity in either cell cultures or mice, once again feeding the debate concerning the detrimental role of iron in DOX-induced cardiotoxicity.

Material and methods

Cell culture and treatments

The H9c2 embryonic rat heart-derived and the EMT-6 mouse mammary carcinoma cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA) (see Supplementary material online for details). All cell lines were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 4 mM L-glutamine, 100 U/mL penicillin, 100 U/mL streptomycin and 250 ng/mL amphotericin B in humidified air (5% CO₂) at 37 °C. Assays on cells were performed by COHIRO Biotechnology (Faculty of Medicine, Dijon, France).

Briefly, when the cultures reached 70–80% of confluence, cells were suspended in 0.05% (w/v) trypsin/0.53 mM ethylenediaminetetraacetic acid (EDTA) and plated at a density of 5000 cells/well in 96-well plates in medium containing 5% FBS and 2 mM L-glutamine. After 24 h, Iron-Dextran (Ferristat®) was added to achieve the final concentrations of 125, 250, 500 and 1000 μ g/mL. Six hours after iron loading, cells were treated with or without 1 μ M DOX (Adriablastin®) for 20 and 68 h of

treatment for H9c2 cells, and with 2 or 10 μ M of DOX for 20 h of treatment for EMT-6 cells. Cell viability was measured with the XTT assay, which is based on the ability of metabolically active cells to reduce tetrazolium salt XTT (2,3-bis-(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxyanilide inner salt) to orange colored compounds of water-soluble formazan crystals via mitochondrial and extracellular dehydrogenases. The medium was removed and replaced with fresh medium without FBS and L-glutamine containing 0.15 mg/mL XTT solution, and the cells were further cultured in the CO₂ incubator at 37 °C for 4 h. The amount of formazan produced was detected by measuring absorbance at 499 nm, and a reference wavelength was used at 660 nm on an Infinite M200 Pro microplate reader (TECAN, Lyon, France). Cell damage was measured by the release of lactate dehydrogenase (LDH Kit, Promega, Charbonnières, France) into the extracellular medium. Cell viability and cell damage were expressed as a percentage of control, which was taken as 100%. In all experiments, four sets of wells were run and the experiment was repeated at least three times with different cell preparations.

Animals and treatments

Male C57Bl/6 mice (Charles River, L'Arbresle, France) aged 10 weeks were used. All animals received humane care and study protocols complied with the institution's guidelines. The investigation complied with Directive 2010/63/EU of the European Parliament and with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and was approved by the local ethics committee (Comité d'Ethique de l'Expérimentation Animale, Université de Bourgogne, Dijon, France, protocol agreement number: 3211). Throughout the procedure, care was taken to prevent suffering and to ensure animal welfare, for instance, through improving the environment in cages. During the protocol, no animals became moribund and needed to be euthanized early or died from the treatment.

The experimental protocol is described in Fig. 1. To evaluate the effects of IO on DOX-induced cardiotoxicity, 32 male mice had echocardiography at D0; then, for 20 days (D1 to D20), they received a daily intraperitoneal (i.p.) injection of dextran/iron or saline solution. At D21 a second echocardiography was done and the mice were given or not given a single i.p. injection of doxorubicin (DOX, 6 mg/kg). Four groups were then constituted: a control group (CONT, n = 8), iron loading alone (IO, n = 8), DOX alone (DOX, n = 8) or the combination of iron and DOX (IO + DOX). At D30, all of the mice underwent

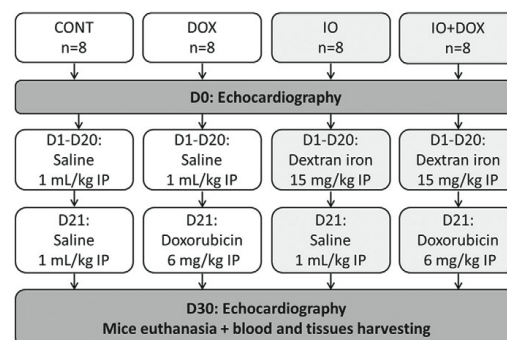


Fig. 1. Experimental design and study groups. At D0, 32 male mice underwent transthoracic echocardiography. From D1 to D20 mice received a daily i.p. injection of dextran/iron (15 g/kg) or saline solution, followed or not at D21, after a second echocardiography, by a single i.p. injection of doxorubicin (DOX, 6 mg/kg). Four groups were then constituted, control groups (CONT, n = 8), iron loading alone (IO, n = 8), DOX alone (DOX, n = 8) or the combination of iron and DOX (IO-DOX). At D30, all mice underwent echocardiography.

echocardiography and were then euthanized (pentobarbital, 80 mg/kg, i.p.) in order to collect blood and tissues.

Transthoracic echocardiographic imaging

Transthoracic echocardiography using the Vevo770™ imaging system (VisualSonics Inc., Toronto, Canada), equipped with a 30 MHz probe was performed at D0, D21 and D30 just before the mice were euthanized. Briefly, the mice were anesthetized with isoflurane. The body temperature of the mice was monitored using a rectal probe, and an infrared heating lamp was used to maintain body temperature at 37 ± 0.5 °C throughout the procedure. An electrocardiogram (ECG) signal was also monitored through electrode pads on the heated platform. Chest hair was removed using a chemical depilator to minimize ultrasound attenuation. The ultrasound probe (RMV-707B) was placed on the chest of the mice using warm ultrasound gel as a coupling medium.

Two-dimensional images were recorded in the parasternal long- and short-axis views to guide M-mode recordings obtained at the mid-ventricular level, with 3 to 5 measurements for each view and the average was calculated. The thicknesses of the inter-ventricular septum (IVS) and left ventricular posterior wall (LVPW), as well as the left ventricular (LV) end diastolic and end systolic internal dimensions (LVEDD and LVESD, respectively) were measured, and the average values were calculated. LV systolic function was computed from the M-mode measurements according to the recommendations of the American Society of Echocardiography Committee (Sahn et al., 1978). Left ventricular mass normalized to body weight (LVMN) was calculated using the formula:

$$\text{Echo LV mass} = (VST_d + LVID_d + LVPW_d)^3 - (LVID_d)^3 / \text{body weight (g)}.$$

Pulsed Doppler studies of LV diastolic function were performed in the apical 4-chamber view with the Doppler cursor oriented parallel to the long-axis plane of the left ventricle. The sample volume was placed just below the level of the mitral valve and adjusted to render the highest early diastolic flow velocity peak of the transmitral Doppler flow signal. There was often a need to correct the angle, which was always less than 20°. The early and late diastolic peak velocities (E, A) and their ratio (E/A) were derived from the transmitral Doppler waveform. Left ventricular systolic intervals of the isovolumic contract time (IVCT), the ventricular ejection time (ET) and diastolic intervals of the isovolumic relaxation time (IVRT) were also derived from the transmitral Doppler waveform. The myocardial performance index (Tei index) was calculated using the formula:

$$\text{Tei index} = (IVCT + IVRT) / ET.$$

Blood sampling and tissue processing

At D30 after the echocardiography, the animals were anesthetized with sodium pentobarbital (80 mg/kg, i.p.) until total loss of nociceptive reflexes (verified by paw pinching), and heparin was injected (500 IU/kg, i.p.). Blood was collected from the heart, and immediately centrifuged at 4 °C in order to separate the plasma. The heart, liver, epididymal fat and kidneys were quickly harvested and immediately frozen in liquid nitrogen. Samples were then stored at -80 °C until subsequent measurements.

Measurement of iron content

Iron content was measured in non-hemolysed plasma, and in the myocardium, fat and liver from all animals using atomic absorption spectroscopy in the University facility (Welience, Analytical Chemistry Pole, Faculty of Pharmacy, Dijon, France). Plasma and tissues were

mineralized in a mixture of concentrated acids. Total iron content was determined using atomic absorption spectrometry in a graphite furnace. Total iron was established using the ContrAA 700 (Analytik Jena, Germany), a high-resolution continuous-source atomic absorption spectrometer, at the wavelength of 248.3270 nm.

Oxidative stress measurements

RONS in the heart were measured using electron spin resonance (ESR) spectroscopy (Vergely et al., 2003): freshly-harvested hearts were perfused with ice-cold saline solution to remove residual blood. Myocardial biopsies of 2.5 mm diameter were excised, rinsed, weighed and placed in a 24-well tissue culture plate containing 200 µL of ESR-specific pH 7.4 Krebs-HEPES buffer, comprising (in mM) Na-HEPES 20; NaCl, 99; NaHCO₃, 25; KCl, 4.69; MgSO₄, 1.2; KH₂PO₄, 1.03; CaCl₂, 2.5; and glucose, 5.5; to which deferoxamine (25 µM) and diethylthiocarbamate (5 µM) were added to remove traces of contaminant metal ions. The 1-hydroxy-3-methoxy-carbonyl-2,2,5,5-tetramethylpyrrolidine (CMH) spin probe was added in order to reach a final concentration of 100 µM; then the tissue was incubated for 15 min at 37 °C, in a 5% CO₂ atmosphere, in the presence of CMH. In the presence of RONS, the ESR-silent CMH hydroxylamine is oxidized into CP' nitroxide radical with a characteristic 3-line signal. The incubation medium and tissue were then collected and immediately frozen in liquid nitrogen in ESR-compatible polyethylene tubes. The frozen tissue and medium were analyzed at 100 K in a Bruker X-band EMX spectrometer (Bruker, Wissembourg, France) using an HS cavity, with the following recording parameters: amplitude modulation 5 G, frequency modulation 100 kHz, gain 2.10^{+5} , time constant 328 ms, conversion time 40.96 ms, microwave frequency 9.37 GHz, field sweep 100 G, attenuation 10 dB.

The height of the central anisotropic peak of the CP' signal was measured (in arbitrary units, AU) and results were expressed in AU/mg of fresh tissue.

Myocardial gene expression

Total RNA was extracted from heart samples using Qiazol reagent (Invitrogen Life Technologies, Saint-Aubin, France). The quality and concentration were checked using Agilent technology.

mRNA (1 µg) was reverse transcribed with Moloney murine leukemia virus (MMLV) reverse transcriptase (Invitrogen, id.) using random hexamers (Invitrogen, id.) according to the manufacturer's instructions. Real-time quantitative polymerase chain reaction (RT-PCR) was performed with 2 µL of cDNA using the SYBR-Green PCR Master-Mix (Applied Biosystems) and both sense and antisense primers (5 µM) in a final volume of 20 µL, in an 7500-Standard-Real-Time PCR system (Applied Biosystems). Cardiac mRNA levels of beta-myosin heavy chain (β-MHC), atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), catalase, manganese- and extracellular superoxide dismutase (Mn-SOD and Ec-SOD). Target gene expression was normalized against the housekeeping hypoxanthine-guanine phosphoribosyltransferase (HPRT) gene expression. The abundance of each target mRNA was normalized to that of HPRT mRNA. The primers used for the amplification of mouse genes are provided in Table 1.

Statistical methods

Continuous data were expressed as means (standard deviations) or medians (25th percentile–75th percentile) and dichotomous data as numbers (percentages). For continuous variables, the normality of distribution was tested by the Shapiro–Wilk test.

At day 30, because of the presence of two factors in the treatment groups, all other continuous data were compared using 2-way ANOVA. This allowed us to analyze the effects of IO and DOX alone and the potential interactions between these two factors. Post-hoc analyses

Table 1
Forward and reverse sequences of primers used for the amplification of mouse genes.

	Forward (5' to 3')	Reverse (5' to 3')
HPRT	CTGGTGAAAGGACCTCTCG	TGAAGTACTCATTATAGTCAAGGGCA
ANP	GAGAAGATGCCGGTAGAAGA	AAGCACTGCCGTCTCTCAGA
BNP	ATGGATCTCTGAAGGTCTG	GTGCTGCTTGAGACCGAA
β-MHC	GCCAAACCAACCTGTCAAGTTC	TGCAAAGGCTCCAGGTCTGAGGGC
Catalase	GATGAAGCAGTGAAGGAGC	ACCACATCTGAACGAGGAG
Ec-SOD	CTGCTGCTACATAACAGC	ATGGCTGAGGTTCTCTGCAC
Mn-SOD	AACTCAGGTCGCTCTCAGC	GCTTGATAGCCTCCAGCAAC

were done with Tukey's test if appropriate. Analyses were done using Sigma Plot 12.0 (Systat software, USA).

Results

Effect of DOX, in combination with dextran–iron, on the viability of cultured cells

In a series of preliminary *in vitro* experiments, the effects of different concentrations of DOX on cell viability (XTT test) and cellular damage (release of LDH) were tested in cultures of cardiomyocytes (H9c2) and cancer cells (EMT-6). At 1 μM concentration, DOX was able to reduce H9c2 cell viability by about 40% after 20 h of exposure (Fig. 2A), and by about 90% after 68 h of exposure (Fig. 2C); at this time point, the release of LDH into the culture medium was increased by 50% (Fig. 2D), showing that cell damage had been induced. The anti-proliferative and cytotoxic effects of DOX were not reinforced by iron pre-treatment. Conversely, for the highest doses (500 and 1000 μg/mL), dextran–iron was able to increase cell viability (Fig. 2A and C) and even reduced LDH release for the longest exposure of 68 h (Fig. 2D).

The same experiment was repeated in EMT-6 cancer cells, but since these cells have a very high growth rate and rapidly attain confluence, we decided to challenge them with higher concentrations of DOX (2 and 10 μM), instead of using longer exposure. 2 and 10 μM of DOX were able to reduce cell viability by about 20% and 60%, respectively (Fig. 2E and G), and increased LDH release into the extracellular medium by a factor of more than 2 and 10, respectively (Fig. 2F and H). Iron pre-treatment alone did not modify cell viability (data not shown); however, pre-treatment with 125 or 250 μg/L of dextran–iron increased the anti-proliferative effect of 10 μM of DOX on EMT-6 cancer cells (Fig. 2G).

We therefore aimed to transpose these results obtained *in vitro* to an *in vivo* model.

Iron levels in plasma and tissue

Iron levels were evaluated in the plasma and in several tissues at D30 (Fig. 3), in order to validate the IO model in mice, and to observe possible relationships between DOX treatment and the iron status of tissues. In the plasma, 20 days of daily dextran–iron injections led to an increase in circulating iron only in the IO-DOX group. Myocardial iron content in the IO group had more than doubled while levels in the single DOX injection group had not increased significantly. Moreover, treatment with DOX induced a decrease in myocardial iron content in IO mice ($p = 0.049$). Pretreatment with iron led to a more than 6-fold increase in liver iron content ($p < 0.001$), while the injection of DOX in the context of IO led to a smaller increase in liver iron content ($p = 0.025$). In epididymal fat, iron loading led to dramatic increases in iron content in both IO and IO-DOX groups, but DOX decreased iron content in IO-DOX mice ($p = 0.025$). Overall, the results confirmed the presence of IO in all investigated tissues and showed interplay between DOX and iron. Indeed, DOX tended to decrease iron content and/or to accelerate the elimination of iron from these tissues, except for the plasma where the opposite effect was observed. We therefore investigated the myocardial repercussions of IO and DOX on myocardial function, oxidative stress and gene expression.

Myocardial dysfunction

Measurement of left ventricular function by echocardiography. Because of physiological variations in LV parameters in mice, each mouse was compared with itself at baseline, D21 and D30 (Table 2). The percentage of variation compared with baseline values was calculated: namely the change in left ventricular ejection fraction (Δ LVEF) and in left ventricular mass (Δ LVMN) (Fig. 4A and B, respectively).

Twenty days of daily injections of dextran–iron (15 mg/kg) did not modify LVEF at D21 but induced significant left ventricular hypertrophy ($p = 0.031$). At D30, there was a significant decrease in systolic function assessed by a fall in LVEF in the DOX ($44\% \pm 7$ vs. $62\% \pm 5$, $p < 0.001$) and IO-DOX ($51\% \pm 5$ vs. $63\% \pm 9$, $p = 0.012$) groups as compared with their baseline values. A significant increase in LVMN was still present in the IO group at D30 ($p = 0.013$), as compared with baseline and with values for other groups; moreover, a significant interplay between iron and DOX was observed, in which DOX decreased LVMN in IO-DOX mice ($p = 0.004$).

Concerning left ventricular diameters, there was a significant increase in LVEDD in the DOX group compared with baseline (4.06 ± 0.25 mm vs. 3.67 ± 0.35 mm, $p = 0.007$), but not in the IO-DOX group. There was no significant difference among groups for all of the diastolic parameters (Δ E/A, Δ IVRT, Δ Tei index).

Collectively, these results confirm that IO alone did not impair left ventricular function, and that IO did not exacerbate DOX-induced impairment of LVEF. We therefore aimed to link these functional results to cellular markers of hypertrophy, heart failure and oxidative stress.

Cellular markers of hypertrophy and heart failure: myocardial mRNA expression of ANP, BNP and β-MHC. There were significant differences among groups regarding myocardial expression of ANP, BNP, and β-MHC assessed by qRT-PCR (Fig. 5). At D30, the expressions of ANP (16.50 ± 5.71 vs. 7.35 ± 3.62 , $p = 0.004$, AU) and β-MHC (0.77 ± 0.18 vs. 0.35 ± 0.14 , $p = 0.014$, AU) were increased by DOX. Additionally, IO in mice treated with DOX further increased the expression of BNP (27.15 ± 6.70 vs. 18 ± 4.99 , $p = 0.025$, AU) and β-MHC (1.09 ± 0.26 vs. 0.77 ± 0.18 , $p = 0.048$, AU), but not ANP.

Oxidative stress measurements

Measurement of RONS in the heart using ESR spectroscopy. We found a positive effect of iron loading on the cardiac production of RONS, with a significantly higher CP' signal intensity in the IO group than in the CONT group ($p = 0.036$), and in the IO-DOX group than in the DOX group ($p = 0.041$) (Fig. 6A).

Myocardial expression of anti-oxidant enzyme. The expression of the antioxidant enzymes catalase and Ec-SOD was greater in the IO group ($p = 0.001$) than in the CONT group. Curiously, the injection of DOX in mice pretreated with iron attenuated the increase in the expression of catalase ($p = 0.007$) and Ec-SOD ($p = 0.005$). No modifications in Mn-SOD expression were induced by the different treatments.

These results led us to the conclusion that IO induced an increase in the production of reactive oxygen/nitrogen species in the myocardial tissue, which was then compensated for by the up-regulated expression of antioxidant enzymes. This mechanism may counterbalance the deleterious redox effects of DOX.

Discussion

The first aim of our study was to evaluate the effects of the combination of iron and DOX on cell viability in two cell lines: EMT-6 cancer cells and H9c2 cardiomyocytes. While 1 μM of DOX induced a significant reduction in H9c2 and EMT-6 cell viability, pretreatment of these cells with dextran–iron (125–1000 μg/mL) exerted distinct effects: the highest dextran–iron concentrations reinforced DOX cytotoxicity in

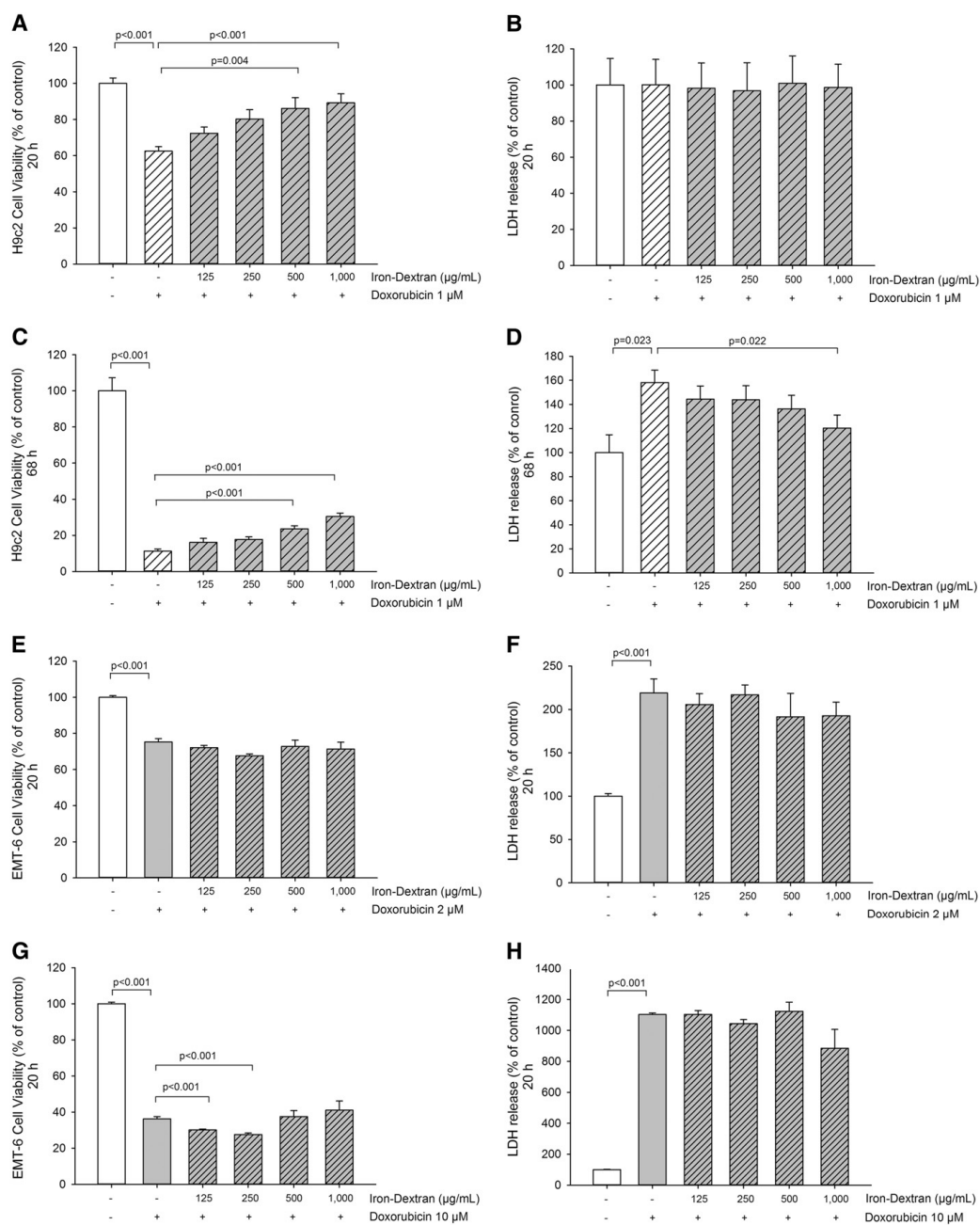


Fig. 2. Cell viability (assessed by XTT assay: A, C) or cell damage (assessed by LDH release: B, D) in H9c2 cardiomyocytes incubated in the presence of increasing concentrations of iron-dextran with 1 μM of DOX for 20 h (A, B) or 68 h (C, D). Cell viability (E, G) and cell damage (F, H) in EMT-6 cells incubated for 20 h in the presence of increasing concentrations of iron-dextran with 2 μM (E, F) or 10 μM (G, H) of DOX. Values are means ± SEM. Differences among groups were assessed by one-way ANOVA with a Tukey test as the post-hoc analysis.

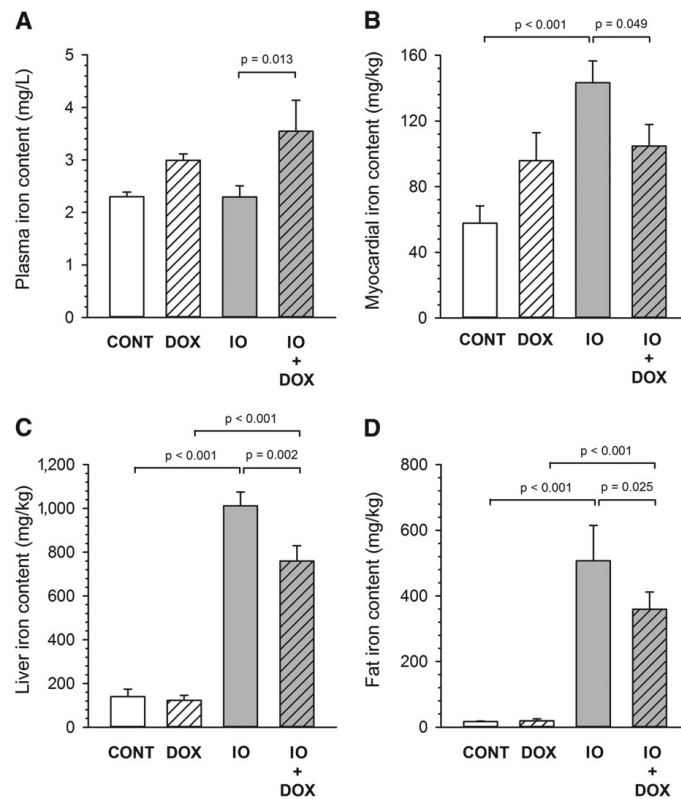


Fig. 3. Iron content: assessed by atomic absorption spectroscopy in (A) plasma expressed in mg/L, (B) myocardium, (C) liver and (D) epididymal fat expressed in mg/kg of wet tissue weight, assessed 29 days (D30) after the beginning of daily injections of dextran/iron (1.5 mg/kg from D1–D20) followed by single i.p. injection of DOX (6 mg/kg at D21). Values are means \pm SEM. Differences were assessed using 2-way ANOVA with a Tukey test as the post-hoc analysis.

EMT-6 cancer cells, but protected H9c2 from DOX-induced cell damage. Our results contradict the concept that DOX cytotoxicity is potentiated by iron in cardiac cells. In neonatal rat cardiomyocytes and in H9c2 cardiomyocytes, ferric ammonium citrate (FAC) decreased the viability of cells incubated in the presence of DOX (Link et al., 1998, 1996; Xu

et al., 2008). However, Corna et al. (2004) demonstrated that DOX, in concentrations ranging from 5 to 10 μ M, paradoxically protected H9c2 cardiomyocytes against the toxicity of ferric ammonium citrate. Our study, like theirs, showed that iron did not systematically reinforce the toxic effects of DOX on cardiomyocytes, and did not impair the ability

Table 2
Echocardiographic measurements.

	Baseline (Day 0): Before treatment				Day 30: After treatment			
	CONT	DOX	IO	IO-DOX	CONT	DOX	IO	IO-DOX
n	8	8	8	8	8	8	8	8
HR, bpm	433 \pm 86	405 \pm 86	413 \pm 49	429 \pm 71	387 \pm 43	393 \pm 39	415 \pm 51	413 \pm 61
LVEF, %	60 \pm 4	62 \pm 5	58 \pm 6	63 \pm 9	57 \pm 3	44 \pm 7 [†] *	58 \pm 8	51 \pm 5 [†]
LVEDD, mm	3.76 \pm 0.21	3.67 \pm 0.35	3.90 \pm 0.21	3.87 \pm 0.29	4.03 \pm 0.28	4.06 \pm 0.25 [†]	4.07 \pm 0.29	4.08 \pm 0.42
LV/MN, mg/g	3.19 \pm 0.35	2.87 \pm 0.47	3.16 \pm 0.29	3.37 \pm 0.24	3.36 \pm 0.32 [#]	3.2 \pm 0.40 [#]	3.97 \pm 0.46 [†]	3.47 \pm 0.14 [#]
E wave	539 \pm 144	712 \pm 168	680 \pm 201	691 \pm 91	675 \pm 110	779 \pm 125	848 \pm 289	925 \pm 647
A wave	365 \pm 56	462 \pm 111	381 \pm 107	408 \pm 70	424 \pm 86	472 \pm 92	453 \pm 214	605 \pm 604
E/A ratio	1.70 \pm 0.17	1.66 \pm 0.35	2.0 \pm 0.50	1.72 \pm 0.24	1.58 \pm 0.14	1.78 \pm 0.39	1.97 \pm 0.31	1.76 \pm 0.41
Tei index	0.63 \pm 0.08	0.71 \pm 0.16	0.72 \pm 0.13	0.86 \pm 0.20	0.82 \pm 0.13	0.82 \pm 0.13	0.88 \pm 0.13	0.90 \pm 0.16

Data are means \pm standard deviation. Bpm indicates beats per minute; HR: heart rate; LVEF: left ventricular ejection fraction; LVEDD: left ventricular end-diastolic diameter; LVFS: left ventricular fraction shortening; LV/MN: left ventricular mass normalized to body weight; n: number of mice.

[†] p < 0.01 compared to baseline for each group.

* p < 0.005 compared to CONT group at day 30.

[#] p < 0.05 compared to IO group at D30 using 2-way ANOVA for repeated measures with a Tukey test as the post-hoc analysis.

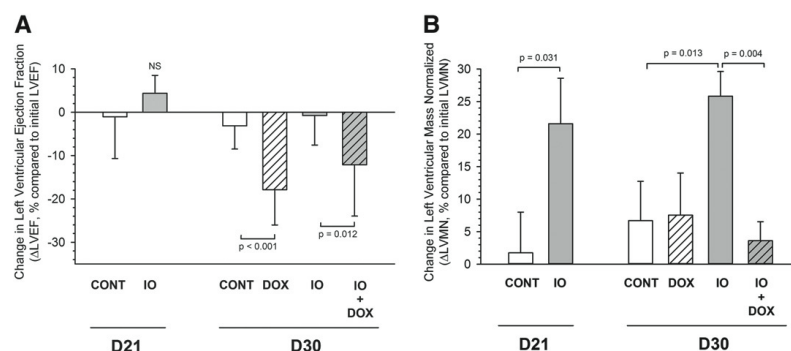


Fig. 4. Left ventricular changes assessed by (A) Δ left ventricular ejection fraction (LVEF) and (B) Δ left ventricular mass normalized to body weight (LVMN). LVEF and LVMN were calculated with 2-dimensional B-mode echocardiography images at baseline (D0), 20 days (D21) or 29 days (D30) after the beginning of daily injections of dextran/iron (15 mg/kg from D1–D20) followed by a single i.p. injection of DOX (6 mg/kg, D21). LVMN was used as a measure of cardiac hypertrophy. Values are mean \pm SEM. Differences among groups were assessed by 2-way ANOVA with a Tukey test as the post-hoc analysis.

of DOX to alter cell viability in cancer cell lines. We therefore aimed to determine whether these results obtained *in vitro* were replicated in an *in vivo* model.

Interestingly, DOX induced differences in iron tissue distribution after a total cumulative dose of 300 mg/kg of iron-dextran. Iron loading increased the concentration of iron in the heart, liver and fat; however, since the assay was performed ten days after cessation of the iron-

dextran injections, increased levels were not observed in the plasma where clearance occurs first. Remarkably, the administration of DOX increased iron concentrations in the plasma, while iron content of the heart, liver and fat was significantly decreased. We can only speculate on the reasons for this difference, but our results are in accordance with those obtained in rats (Panjra et al., 2007) and mice (Moon et al., 2011) and confirm that iron mainly accumulates in the liver and

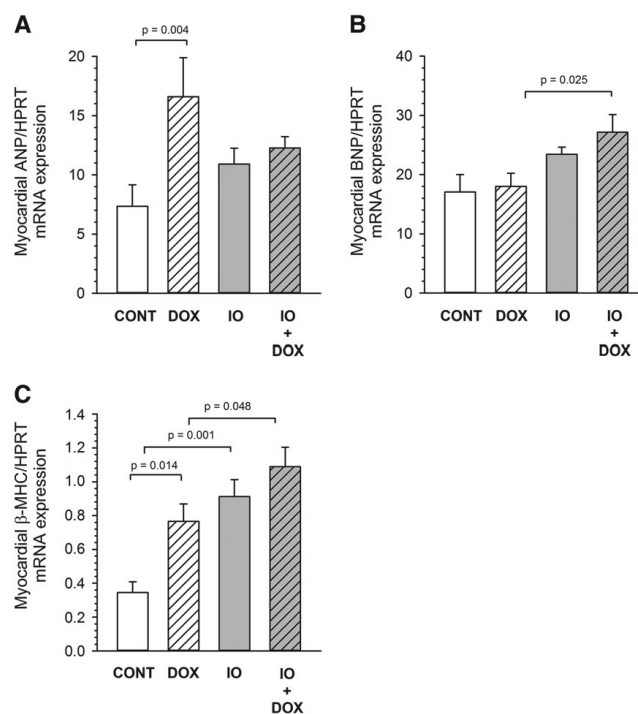


Fig. 5. Myocardial mRNA expression of (A) atrial natriuretic peptide (ANP), (B) brain natriuretic peptide (BNP) and (C) β -myosin heavy chain (β -MHC) related to that of hypoxanthine-guanine phosphoribosyltransferase (HPRT), in arbitrary units (AU), 29 days (D30) after the beginning of daily injections of dextran/iron administration (15 mg/kg from D1–D20) followed by single i.p. injection of DOX (6 mg/kg, D21). Values are means \pm SEM. Differences among groups were assessed by 2-way ANOVA with a Tukey test as the post-hoc analysis.

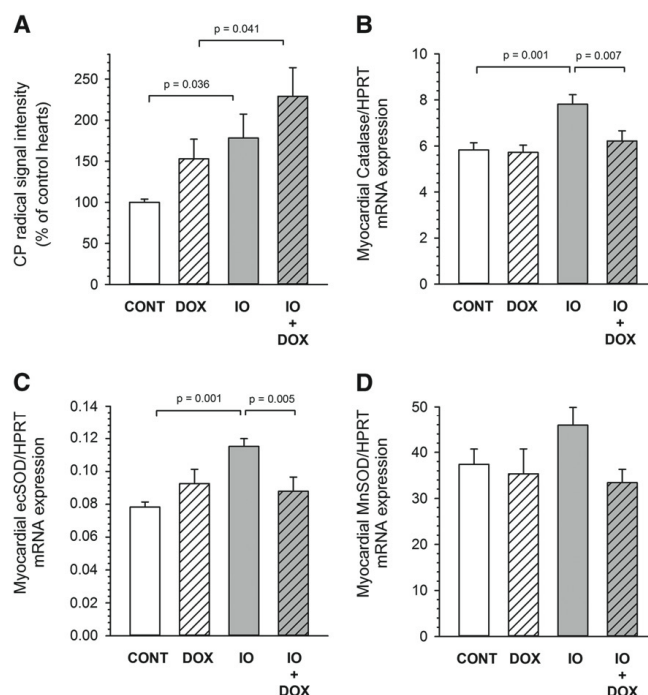


Fig. 6. Myocardial oxidative stress. (A) Measurements of myocardial oxidative stress using ESR spectroscopy with CMH as a spin probe, converted to the CP^{\bullet} radical in the presence of reactive oxygen–nitrogen species RONS; (B) myocardial mRNA expression of (B) catalase, (C) extracellular superoxide dismutase (Ec-SOD) and (D) manganese-superoxide dismutase (Mn-SOD) related to this of hypoxanthine–guanine phosphoribosyltransferase (HPRT), in arbitrary units (AU), 29 days (D30) after the beginning of daily injections of dextran/iron (15 mg/kg from D1–D20) followed by a single i.p. injection of DOX (6 mg/kg, D21). Differences were assessed by 2-way ANOVA with a Tukey test as the post-hoc analysis.

fat, while accumulation in other tissues, including the heart, occurs at a lower level, and that accumulated iron may be susceptible to pharmacological interactions with DOX. It has been demonstrated that hepcidin is expressed in the heart; this implies that it might play a role in iron homeostasis in the heart. In pathological conditions, such as heart failure and myocarditis, the increased expression of hepcidin could induce iron mobilization from the heart cells (Rochette et al., 2014). In rats, Panjra et al. found no significant effect of DOX on myocardial iron content 2 to 3 weeks after treatment (intravenous 6 mg/kg single dose of DOX) (Panjra et al., 2007). However, Miranda et al. found a significant increase in iron content in the plasma and in the liver, spleen and heart of wild-type female mice 4 days after a single 20 mg/kg dose of DOX (Miranda et al., 2003). These conflicting results could be partially explained by the three-fold higher dose of DOX used in these previous studies, which could have led to erythrocyte damage (Myers et al., 1982) and thus induced the release of iron from hemoglobin, as suggested by the large amount of iron in the spleen and macrophages.

Concerning heart function, we observed an increase in LVMN, a marker of hypertrophic changes, in the IO group at D21 and D30. The pathological impact of iron overload on myocardial function is complex. Moon et al. established a mouse model of iron overload (IO) (Moon et al., 2011), in which echocardiographic evaluations revealed an increase in wall thickness without alterations in LV systolic function. In the present study, the large increase in echocardiographic LVMN was associated with the up-regulation of cardiac levels of β -MHC mRNA, a marker of myocardial remodeling and hypertrophy (Munoz et al., 2010). Interestingly, our model of IO in mice did not induce heart failure, as proved by the preservation of LV function. In fact,

Yang et al. showed that short durations of IO induced eccentric cardiac hypertrophy and increased cardiac output in Mongolian gerbils, while longer durations of IO led to concentric cardiac hypertrophy and cardiac output impairment (Yang et al., 2002).

This accumulation of iron could trigger an increase in myocardial oxidative stress. In fact, ESR spectroscopy revealed a significantly higher CP^{\bullet} signal intensity in the iron treated groups than in the CONT group. As an adaptive response, the myocardial gene expression of catalase and Ec-SOD was significantly increased in the IO group. Bartfay et al. observed that chronic iron overload in mice resulted in free radical-mediated alterations of cardiac function, as measured by excess concentrations of cytotoxic aldehyde-derived peroxidation products (Bartfay et al., 1999). Moreover, Al-Rousan et al. described a 100% increase in superoxide abundance and a 114% increase in protein oxidation in the myocardium after 10 weeks of massive iron overload (Al-Rousan et al., 2009). Excess 'labile iron' is potentially detrimental to cells because of its propensity to participate in oxidation–reduction reactions, which generate harmful free radicals (Deb et al., 2009). Given the key catalytic role of free cellular iron in the production of dangerous hydroxyl radical, organisms are equipped with specific proteins designed for iron uptake, transport, and storage, as well as sophisticated mechanisms that regulate intracellular iron homeostasis (Gudjoncik et al., 2014; Simunek et al., 2009). The labile pool of cytosolic iron, which apparently corresponds to iron in transit between the transporter transferrin and the storage protein ferritin, is sensed by iron regulatory proteins (IRPs), which maintain the intracellular free Fe pool at a low level. However, Thomas and Aust showed that DOX may increase the amount of free, redox-active iron by generating superoxide anion, which mediates

the slow reductive release of iron from ferritin (Thomas and Aust, 1986). However, the localization of iron inside the mitochondria seems to be a key event in DOX-induced cardiotoxicity since it was observed recently that mice lacking mitochondrial ferritin are more sensitive to doxorubicin-mediated cardiotoxicity (Maccarinelli et al., 2014) and that the cardiotoxic effects of doxorubicin are linked to the preferential accumulation of iron inside the mitochondria following doxorubicin treatment (Ichikawa et al., 2014). However, in our experimental conditions, the increase in myocardial oxidative stress was accompanied by the up-regulation of antioxidant gene expression, which may have played a counterbalancing role in this situation. It is not yet known, however, if this up-regulation of antioxidant enzyme expression is only mediated through increased myocardial ROS production or if iron response elements could post-transcriptionally control the induction of antioxidant enzyme genes.

Concerning the effects of a single intra-peritoneal dose of 6 mg/kg of DOX in mice, at D30, there was a fall in LVEF and a significant increase in LVEDD in the DOX group as compared to baseline values, but no mice died following the treatment. Indeed, LV systolic function impairment is a well-known side-effect of DOX in humans (Singal and Iliskovic, 1998). In mice, Walker et al. found similar results 10 days after a single dose of 20 mg/kg of DOX (Walker et al., 2011). Interestingly, the dose of DOX that we used in our study, which did not lead to any deaths, may be regarded as mild as compared to the usual doses used to induce acute cardiac toxicity in rodents. However, this single injection was able to induce alterations in cardiac contractility in the DOX group and to up-regulate the mRNA expression of ANP and β -MHC in heart tissue. Regarding natriuretic peptides, our results are in accordance with those of Huelsenbeck et al.: 2 days after a single dose of 10 mg/kg in mice, DOX caused a large increase in the amount of ANP mRNA, but not BNP mRNA levels (Huelsenbeck et al., 2011). In a previous study carried out in our laboratory, we found that chronic treatment with DOX in rats was able to induce the mRNA expression of β -MHC in ventricular tissue (Richard et al., 2011). Indeed, pathological cardiac dysfunction has been associated with the up-regulation of fetal genes such as ANP (Hystad et al., 2001) and β -MHC. ANP is a natriuretic peptide secreted by atrial myocytes under normal conditions. In a failing heart, ventricular myocytes also secrete ANP (van den Bosch et al., 2006). The expression of the α and β isoforms of MHC genes is developmentally regulated in the myocardium (Lompre et al., 1991). One consistent molecular marker of DOX-induced cardiac maladaptation is the up-regulation of β -MHC with the corresponding down-regulation of α -MHC. Therefore, a relationship exists between the decreased contractile performance and the relative increase in β -MHC expression. Increased levels of cardiac ANP mRNA and β -MHC mRNA in the DOX group in our study, along with alterations in ventricular contractility, measured using echocardiography, are in accordance with this pathophysiological mechanism and can be linked to DOX-induced cardiomyopathy.

Finally, we set out to determine whether prior IO could potentiate DOX cardiotoxicity. Unlike the DOX group, the IO-DOX group did not develop significant left ventricular dilation compared with baseline values, and at D30, LVEF was not statistically different from that of the CONT group at the same time (Table 1). Thus, the LV functional impairment observed in the IO-DOX group was slightly reduced by prior IO when compared with the DOX group. The cardiac mRNA levels of ANP, an indicator of heart injury, were in accordance with this finding: there was a statistically significant interplay between iron and DOX that prevented the overexpression of myocardial ANP. These data highlight the role of iron in myocardial remodeling and hypertrophy as previously discussed. Our findings contradict the conclusions of Panjrath et al., who found that oral IO in rats potentiated DOX cardiotoxicity, assessed by an increase in *ex-vivo* myocardial ^{99m}Tc annexin-V uptake (Panjrath et al., 2007). However, the present study cannot be compared to the Panjrath study because of marked differences in cardiac evaluation and the lack of direct markers of heart failure in the Panjrath study. Miranda et al.

found a greater degree of mitochondrial damage in the hearts of *Hfe*^{−/−} female mice undergoing chronic DOX treatment than in the hearts of wild-type mice. Cardiac function was not evaluated in this study, but the authors concluded that these mice showed increased sensitivity to the cardiotoxic effects of DOX using mortality rates (Miranda et al., 2003). However, given the very high doses of DOX delivered in the Miranda study (20 mg/kg), and the absence of direct proof of myocardial injury, this higher mortality rate could also be linked to systemic damage induced by DOX. Moreover, until now, the role of IO in DOX cardiotoxicity had never been assessed using *in vivo* functional measurements such as echocardiography.

Our findings can be linked to those of Corna et al., who reported a paradoxical benefit of anthracycline-derived reactive oxygen species, increased ferritin synthesis and resistance to iron-mediated damage (Corna et al., 2004). However, in our study, DOX was given after the IO, and our results suggest that prior IO could have partially reduced sub-acute DOX-induced myocardial damage, in the same way that prior stress, like hyperthermia (Ito et al., 1999) or endurance training (Ascensao et al., 2005) reduces myocardial damage. Moreover, Metzler et al. observed that two weeks of iron pretreatment in mice was beneficial in the context of *in vivo* myocardial ischemia/reperfusion injury (Metzler et al., 2007): troponin T levels, as a marker of myocardial damage, histological infarction area and damage score were significantly lower in iron-treated animals. Munoz et al., in an elegant *in vitro* study, proved that the effects of iron on cardiomyocytes could be visualized as a biphasic response: a low concentration of iron was beneficial to cardiomyocytes because it promoted hypertrophy and enhanced survival mechanisms through RONS reduction (Munoz et al., 2010).

Several non-iron-mediated mechanisms could explain why the cardiotoxic action of DOX may affect the heart independently of any interactions with iron. In a recent work performed in mice whose cardiomyocytes were specifically deleted for topoisomerase-II β , Zhang et al. suggested that DOX-induced cardiotoxicity was mediated by topoisomerase-II β inhibition, which leads to transcriptome changes in cardiomyocytes (Zhang et al., 2012). These changes are responsible for defective mitochondrial biogenesis and ROS formation. Additionally, some of the beneficial effects observed with dexrazoxane may be related to its ability to catalytically inhibit topoisomerase II, rather than its iron-chelation activity (Vavrova et al., 2013).

Limitations. The use of more sensitive measurements of cardiac function, such as pressure–volume analysis or conscious echocardiography, could have uncovered previously hidden cardiac dysfunction and may have shed light on the physiological response to iron loading in this model. Moreover, the hypothesis of a preconditioning phenomenon induced by IO could have been strengthened by the use of a chronic model of DOX-cardiotoxicity using weekly DOX injections or by extending the follow-up after a single DOX injection so that repeated echocardiograms could be done before killing the animals. Actual heart weights were not measured to confirm the increase in LV mass as assessed by echocardiography in the IO group. However, it is widely accepted that echocardiography is sensitive enough to assess myocardial hypertrophy and heart functional changes in mice (Liao et al., 2002).

Conclusion

In our experimental conditions, IO alone in mice induced cardiac hypertrophy and increased myocardial oxidative stress levels, and IO followed by an injection of DOX did not result in a significant increase in cardiac impairment evaluated at both the functional and molecular levels. On the contrary, our results even suggest that prior chronic oxidative stress induced by IO could mitigate DOX cardiotoxicity through a “preconditioning” phenomenon that has not yet been elucidated. Additionally, in cardiomyocytes and cancer cell lines, iron exerted distinct effects: the highest dextran–iron concentrations reinforced DOX cytotoxicity in EMT-6 cancer cells and protected H9c2 from DOX-induced

cell damage. Therefore, these results suggest that the role of iron in anthracycline-induced cardiotoxicity may be more complex than currently believed, and raise doubts about the commonly accepted concept of the iron-driven oxidative alterations involved in the cardiotoxic effects of DOX.

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Compliance with ethical standards

Disclosures

On behalf of all authors, the corresponding author states that there is no conflict of interest.

Animals

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

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B. Overweight in mice induced by perinatal programming exacerbates doxorubicin and trastuzumab cardiotoxicity

Objectifs

Plusieurs facteurs de risque cardiovasculaires sont associés au développement de la cardiotoxicité aux anthracyclines +/- trastuzumab. Cependant, en ce qui concerne le rôle de l'obésité et du surpoids, les données de la littérature sont rares. L'objectif de cette étude expérimentale était donc d'élucider l'impact d'un surpoids modéré, induit par une suralimentation post-natale, sur le développement de la cardiotoxicité aux anthracyclines +/- trastuzumab chez la souris.

Résultats

Après une étape de mise au point, nous avons développé un modèle de cardiotoxicité modérée induite par l'association doxorubicine et trastuzumab, permettant l'étude ultérieure de facteurs potentiateurs de celle-ci. Par ailleurs, notre équipe a développé au cours des dernières années un modèle de souris présentant un surpoids associé à un risque cardio-métabolique accru à l'âge adulte, induits par un mécanisme de programmation périnatale par réduction de la taille des portées (suralimentation postnatale). Dans ce modèle, les souris adultes (4 mois) présentent un surpoids de l'ordre de 15% comparé aux souris témoins.

Les souris en surpoids et les souris témoins ont été traitées par une injection intrapéritonéale unique de doxorubicine (6 mg/kg), trastuzumab (10 mg/kg) ou par l'association des deux traitements. La cardiotoxicité était évaluée par échocardiographie à J10 et J20 et par mesure de l'expression myocardique précoce à J3 des gènes de l'atrial natriuretic peptide (ANP), du brain natriuretic peptide (BNP) et de la β -myosin heavy chain (β -MHC)

Comparées aux souris témoins, les souris en surpoids développaient une altération de la fonction systolique cardiaque lors d'une injection unique de doxorubicine. Le trastuzumab seul n'engendrait pas d'altération cardiaque fonctionnelle significative dans les deux groupes. En cas de traitement combiné doxorubicine et trastuzumab, les deux groupes développaient une cardiotoxicité mais avec quelques différences : chez les souris témoins, l'altération de la

FEVG se faisait plus tardivement (J20) et sans dilatation du ventricule gauche à l'inverse des souris en surpoids. En ce qui concerne l'expression myocardique des gènes associés à la cardiotoxicité, on observait une augmentation significative de l'expression de l'ANP et du BNP dans les deux groupes traités par doxorubicine + trastuzumab. En revanche, seul le groupe en surpoids à risque cardio-métabolique accru présentait une augmentation de l'expression de β -MHC cardiaque, en faveur d'un remodelage myocardique précoce.

Conclusion

Grâce à un modèle murin de surpoids modéré et de risque cardio-métabolique accru induit par programmation post-natale, nous avons mis en évidence le rôle potentiateur d'une surcharge pondérale sur le développement de la cardiotoxicité aux anthracyclines. La cardiotoxicité du trastuzumab ne semble pas être en revanche majorée par le surpoids. Nos travaux ont également permis de préciser les conditions dans lesquelles existent des potentialisations des effets lors de l'association doxorubicine et trastuzumab. Les modifications fonctionnelles induites par cette bithérapie sont associées à des modifications de l'expression génique de « médiateurs endogènes ».

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Abstract: Background: Trastuzumab (TRZ) is believed to potentiate doxorubicin (DOX) cardiotoxicity, resulting in left ventricular dysfunction. There is some evidence that overweight could influence anticancer drug-induced cardiotoxicity, though no study has evaluated the impact of moderate overweight, induced by postnatal nutritional programming, on the cardiotoxic effects of DOX alone or in combination with TRZ.

Methods: Immediately after birth, litters of C57BL/6 mice were either maintained at 9 pups (normal litter, NL), or reduced to 3 (small litter, SL) in order to induce programming of ~15% overweight through postnatal overfeeding. At 4 months, NL and SL mice received a single intraperitoneal injection of either saline, DOX (6 mg/kg), or TRZ (10 mg/kg) or both (DOX-TRZ). Transthoracic echocardiography was performed 24 hours before as well as 10 and 20 days after treatments.

Results: Twenty days after DOX administration, systolic dysfunction was observed only in the overweight SL group, while NL mice group had a normal left ventricular ejection fraction (LVEF). However, in the NL group, functional impairment appeared when TRZ was co-administered. Forty-eight hours after drug administration, gene expression of natriuretic peptides (ANP, BNP) appeared to be potentiated in DOX-TRZ mice of both the NL and SL group, whereas the expression of β -MHC increased significantly in overweight-SL mice only.

Conclusions: In an acute model of DOX cardiotoxicity, moderately overweight adult mice were more sensitive to cardiac systolic impairment. Moreover, our results confirm the potentiating action of TRZ on DOX-induced cardiotoxicity in lean mice.

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Overweight in mice induced by perinatal programming exacerbates doxorubicin and trastuzumab cardiotoxicity

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Short title: Overweight exacerbates DOX/TRZ cardiotoxicity

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Brief Summary: This study evaluated the impact of moderate overweight, induced by postnatal nutritional programming, on the cardiotoxic effect of DOX alone or in combination with TRZ. We showed that moderately overweight adult mice were more sensitive to cardiac systolic impairment and confirmed the potentiating action of TRZ on DOX-induced cardiotoxicity in lean mice.

ABSTRACT

Background: Trastuzumab (TRZ) is believed to potentiate doxorubicin (DOX) cardiotoxicity, resulting in left ventricular dysfunction. There is some evidence that overweight could influence anticancer drug-induced cardiotoxicity, though no study has evaluated the impact of moderate overweight, induced by postnatal nutritional programming, on the cardiotoxic effects of DOX alone or in combination with TRZ.

Methods: Immediately after birth, litters of C57BL/6 mice were either maintained at 9 pups (normal litter, NL), or reduced to 3 (small litter, SL) in order to induce programming of ~15% overweight through postnatal overfeeding. At 4 months, NL and SL mice received a single intraperitoneal injection of either saline, DOX (6 mg/kg), or TRZ (10 mg/kg) or both (DOX-TRZ). Transthoracic echocardiography was performed 24 hours before as well as 10 and 20 days after treatments.

Results: Twenty days after DOX administration, systolic dysfunction was observed only in the overweight SL group, while NL mice group had a normal left ventricular ejection fraction (LVEF). However, in the NL group, functional impairment appeared when TRZ was co-administered. Forty-eight hours after drug administration, gene expression of natriuretic peptides (ANP, BNP) appeared to be potentiated in DOX-TRZ mice of both the NL and SL group, whereas the expression of β -MHC increased significantly in overweight-SL mice only.

Conclusions: In an acute model of DOX cardiotoxicity, moderately overweight adult mice were more sensitive to cardiac systolic impairment. Moreover, our results confirm the potentiating action of TRZ on DOX-induced cardiotoxicity in lean mice.

Keywords: Doxorubicin, trastuzumab, cardiotoxicity, overweight, postnatal programming

1. Introduction

Doxorubicin (DOX, Adriamycin®) is an anthracycline with a broad spectrum of activity in oncological practice¹; however, its use is limited by its dose-dependent cardiotoxicity, which may result in both acute and chronic cardiotoxic effects²⁻³. Following the administration of anthracyclines in the adjuvant settings of breast cancer, trastuzumab (TRZ), a humanized monoclonal antibody against the human epidermal growth factor receptor 2 (HER 2) protein⁴, is effective in patients that overexpress this receptor. The association of TRZ and chemotherapy provides a clinical benefit in terms of recurrence-free survival and overall survival compared with chemotherapy alone⁵⁻⁸. Nonetheless, while preclinical studies did not reveal cardiotoxicity⁹, later clinical studies showed that treatment with TRZ led to an unexpected incidence of cardiac side effects¹⁰. The most frequent effect was reduced systolic ventricular function, which was asymptomatic or associated with heart failure¹¹.

In fact, clinical trials have shown a greater incidence of cardiac dysfunction when anthracycline chemotherapy was associated with TRZ than with chemotherapy alone¹⁰ and TRZ is now believed to potentiate the cardiotoxic effects of anthracyclines^{6, 7, 12}. In *in vivo* murine models that combined DOX with TRZ, a synergetic interaction leading to deterioration in cardiac function¹³ and to an increase in cardiomyocyte apoptosis was reported^{14, 15}. In addition, *in vitro* in cardiomyocytes, the blockage of HER2 was accompanied by apoptotic processes and by an increase in the level of oxidative stress possibly through mitochondrial and reactive oxygen species-dependent pathways^{16, 17}.

Several factors may increase the incidence of cancer in overweight and obese individuals¹⁸. Although it has been reported for many years that patients with metabolic syndrome have a higher risk of cardiovascular disease¹⁹, their susceptibility to chemotherapy-induced cardiac disease has only recently been documented. In fact, few studies have shown that obese patients²⁰ or rats fed with a high-lipid diet²¹ are more sensitive to the cardiotoxic effects of anthracyclines. Moreover,

obesity has recently been associated with a poor outcome in breast cancer patients treated with DOX-based chemotherapy^{22, 23}, a finding that could in part be related to greater sensitivity to the side-effects of anticancer agents. Therefore, it is important to explore more deeply the influence of overweight and obesity as aggravating factors in the cardiotoxic effects of anticancer drugs, particularly when several types of chemotherapy are combined.

We recently demonstrated that overfeeding during the immediate postnatal period in mice induced perinatal programming of cardio-metabolic risk. Indeed, postnatal overfeeding not only induced permanent moderate overweight, but also, during adulthood, increased blood pressure, glucose intolerance and insulin-resistance²⁴. This rodent model, which presents several features of mild metabolic syndrome, is also characterized by greater susceptibility to myocardial ischemia-reperfusion injury^{25, 26}. Therefore, this model is potentially relevant to study the impact of overweight on heart sensitization to the cardiotoxic effects of anticancer drugs, and particularly the association of anthracyclines with TRZ.

Thus, the aim of our study was to evaluate in mice the role of overweight in the cardiac impact of acute DOX treatment associated or not with TRZ.

2. Material and methods

2.1. Animals

Adult female C57BL/6 mice (Charles River, L'Arbresle, France) were mated with male mice, then housed in individual cages and given water and standard chow *ad libitum* during pregnancy and lactation. On day-2 after birth, male pups were randomly distributed among the mothers and the litter size was adjusted to 9 pups (normal litter, NL group), or reduced to 3 pups in order to induce postnatal-overfeeding (small litter, SL group) as previously described²⁵. The investigation was approved by the local ethics committee (Comité d'Ethique de l'Expérimentation Animale, Université- Bourgogne-Franche-Comté, Dijon, France, protocol agreement number: 00412.03). Throughout the procedure, care was taken to avoid suffering and to ensure animal welfare.

2.2. Treatments

At 4 months of age, considered as adulthood for mice, control groups originating from NL and overweight groups from SL were subjected to different types of treatment.

A preliminary study was conducted to determine the acute dose of DOX that could impair cardiac contractile function in both groups of mice. For this purpose, DOX (Sigma-Aldrich, St Louis, MO) was tested at 0, 6 and 10 mg/kg (intra-peritoneal (IP) single injection) in 29 NL and 28 SL mice. These low-doses of DOX were chosen because of their mild impact on mortality, which is the case for doses up to 15 mg/kg²⁷. All mice underwent transthoracic echocardiography and our results showed that in mice treated with 6 mg/kg DOX, the impairment of systolic function appeared only in overweight SL mice. At 10 mg/kg, DOX induced systolic dysfunction at D10 and D20 in all groups. Therefore, in order to determine the possible deleterious interaction between DOX and TRZ, the dose of 6 mg/kg of DOX was chosen for the following experiments.

In order to assess the impact of overweight on the development of cardiotoxicity by DOX, TRZ or their association, 74 NL mice and 79 SL mice aged 4 months received at D1 a single IP injection of

either saline solution (NaCl 0.9%), or DOX (6 mg/kg), or TRZ (10 mg/kg^{14, 27, 28}) or DOX (6 mg/kg) + TRZ (10 mg/kg). Thus, 8 groups were constituted (Figure 1).

Body weight was measured at baseline, before each echocardiography and before sacrifice. Transthoracic echocardiography was performed 24 hours before (day 0, D0), ten (D10) and twenty (D20) days after the treatments. A subset of 6 mice per group was euthanized 48 hours after the injections (D3), in order to evaluate early circulating and cardiac modifications. At the end of the protocol (D20), all of the animals were euthanized after anaesthesia under pentobarbital (60 mg/kg, IP); blood and tissue were harvested, immediately frozen in liquid nitrogen and stored at -80 C until further analysis.

2.3. *Transthoracic echocardiographic imaging*

Transthoracic echocardiography was performed using the Vevo770™ imaging system (VisualSonics Inc., Toronto, Canada), equipped with a 30 MHz probe as described previously²⁹. Briefly, mice were anesthetized with isoflurane and their body temperature was maintained at 37±0.5°C. An ECG signal was also monitored. The ultrasound probe (RMV-707B) was placed on the mouse's chest. Two-dimensional images were recorded in the parasternal long- and short-axis views. Pulsed Doppler studies of LV diastolic function were performed in the apical 4-chamber view with the Doppler cursor oriented parallel to the long-axis plane of the left ventricle.

2.4. *Quantitative measurement of BNP and cTnI*

Plasma brain natriuretic peptide (BNP) and cardiac troponin I (cTnI) levels were determined with sandwich enzyme immunoassays (Usen, Life science Inc) on the VICTOR3-V multilabel reader (Wallac-PerkinElmer Life Sciences, Courtaboeuf, France) according to the instructions of the manufacturer (Cloud-Clone Corp., Houston, USA).

2.5. *Myocardial gene expression*

Total RNA was extracted from frozen heart samples and real-time quantitative polymerase chain reaction (RT-PCR) was performed as described before²⁵. Cardiac mRNA levels of atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and beta-myosin heavy chain (β -MHC) were normalized against the housekeeping hypoxanthine-guanine phosphoribosyltransferase (HPRT) gene expression. The primers used for the amplification of mouse genes are provided in Table 1.

2.6. *Statistical analysis*

Continuous data were expressed as means \pm standard deviations (SD) and dichotomous data as numbers (percentages). For continuous variables, the normality of distribution was tested by the Shapiro-Wilk test. For body weight and echocardiographic data, values were compared using 2-way ANOVA for repeated measures followed by a Tukey post-hoc analysis. Because of the presence of two factors in treatment groups (SL vs NL and the treatment administered), all other continuous data were compared using 2-way ANOVA. This allowed the analysis of the effects of TRZ and DOX, and the potential interaction of these two factors. Post-hoc analyses were done with Tukey test if appropriate.

3. Results

3.1. *Mice body weight*

At D0, aged 4 months, SL mice were almost 14% heavier than NL mice (32.6 g vs. 28.8 g, $p<0.001$) and this difference persisted throughout the duration of the experiment (Figure 2).

The administration of DOX, alone or in combination with TRZ, induced a transient decrease in body weight at D10 in the SL groups but not in the NL groups (Figure 2B and 2D).

3.2. *Cardiac function in vivo*

The echocardiographic measurements are summarized in Table 2.

At D0, the left ventricular ejection fraction (LVEF) in NL groups was no different from that in SL groups (Table 2). LVEF remained unchanged over time in CONT saline-treated animals (Figure 3A). DOX did not modify the LV systolic function in the NL group (NL DOX) at D10 or D20 (Figure 3B). However, in the SL DOX group, there was a significant decrease in LVEF at D10 ($49\%\pm 7$ vs. $61\%\pm 5$), which persisted at D20 ($47\%\pm 4$). TRZ alone did not alter LVEF in either the NL or SL groups (Figure 3C). The administration of a combined treatment with DOX and TRZ modified LVEF in both groups: at D10, there was a significant decrease in LVEF only in the SL DOX-TRZ ($49\%\pm 4$ vs. $58\%\pm 3$) group but at D20 ($48\%\pm 5$), the systolic function was similarly affected in both NL and SL groups. At D20, LVEF values in the NL DOX-TRZ group were significantly lower than those in the NL DOX group ($52\%\pm 6$ vs. $58\%\pm 3$, $p<0.01$) whereas there was no significant difference between the SL DOX-TRZ and SL DOX groups.

In the DOX and DOX-TRZ groups, left ventricular diameter (LVEDD) in SL mice was higher than that in NL mice at D10 and D20 ($p<0.005$), suggesting more severe cardiac alteration.

In the present study, we did not observe the development of an abnormal diastolic function profile for mice treated with DOX and TRZ (E/A or E/E').

3.3. *Quantitative measurement of plasma cTnI and BNP*

Cardiac troponin I and BNP levels were measured in blood plasma collected by intra-cardiac puncture at D3 and D20. These results showed low or undetectable levels that were similar in all groups of mice (data not shown).

3.4. *Myocardial expression of genes involved in remodelling and heart failure*

The mRNA expression of BNP, ANP and β -MHC were measured in myocardial tissue harvested 48 hours after treatment (D3) or at the end of the experimental protocol (D20).

Our result showed early but not late expression of these genes. At D3, myocardial expression of ANP and BNP was significantly higher in NL and SL groups treated with the combination of DOX and TRZ than in the untreated groups (Figure 4A and 4B). The combined treatment induced significantly higher ANP gene expression than did DOX or TRZ alone (Figure 4B) in both SL and NL groups.

Conversely, an increase in β -MHC expression was only observed in overweight SL groups treated with the combination of DOX and TRZ, but not in lean NL groups nor in mice treated with DOX or TRZ alone (Figure 4C).

Discussion

The main objective of our study was to investigate the role of moderate overweight in mice in the cardiac consequences of DOX treatment, in combination or not with TRZ. The results clearly confirmed, through the evaluation of cardiac function with *in vivo* echocardiography, that overweight mice are more prone to DOX-induced cardiotoxicity than their lean counterparts, as they show earlier impairment of LVEF and increased ventricular diameters. TRZ potentiated the cardiac effects of DOX, as shown by higher myocardial expression of genes involved in heart failure (ANP, BNP), and increased impairment of left ventricular function in lean NL mice. To our knowledge, this is the first study to evaluate, in a mouse model of postnatal programming of “cardio-metabolic risk”, the consequences of anticancer drugs, and to show that overweight individuals are more sensitive to anthracycline cardiotoxicity.

One of the major findings of our study is that moderately overweight SL adult mice present a greater likelihood of developing cardiomyopathy with systolic dysfunction for very low doses of DOX (6 mg/kg), whereas normal weight NL mice did not. For the higher 10 mg/kg dose of DOX, impairment of left ventricular function was observed in all groups at D10 and D20. In our study, in order to acquire multiple echocardiographic images over time, we intentionally used low doses of DOX that did not cause death, but were still able to induce acute cardiac toxicity.

The results presented here suggest that our murine model of moderate overweight, obtained by postnatal programming through over-nutrition during the suckling period, is highly sensitive to DOX-induced cardiotoxicity. To the best of our knowledge, no previous study has shown that moderate overweight could aggravate DOX-mediated cardiotoxicity. Our results could be linked to those of Mitra *et al.*, who reported evidence of increased DOX-cardiotoxicity in a rodent model of obesity²¹. In their work, male rats were obese (30% overweight) after 43-days of a 40% high-fat diet and cardiotoxicity was evaluated after a single IP dose of DOX (8 mg/kg). The results showed

that while only 10% of the normal diet rats died after DOX administration, 80% of the obese rats died, thus exhibiting the increased sensitivity of obese rats to DOX-induced toxicity. Our study, using both functional and molecular cardiac assessments, supports their hypothesis and emphasizes the major role of overweight in DOX cardiotoxicity.

The mechanisms by which obesity is able to promote DOX-induced cardiotoxicity must be specified with further studies. In a study from our laboratory, we reported that overweight 6-month-old rodents had higher cardiac susceptibility to ischemia-reperfusion injury, increased cardiac oxidative stress and metabolic alterations such as increased leptin and decreased adiponectin levels²⁶. From a clinical point of view, obesity is an established risk factor for several cancers³³. Unfortunately, the occurrence of cardiac events during anthracycline-based chemotherapy has almost never been recorded in trials. Thus, clinical data are required to determine whether obese patients are more prone than non-obese patients to the cardiotoxicity of DOX or TRZ treatments.

Our results showed that TRZ could potentiate the cardiotoxicity induced by DOX, especially in the context of lean NL mice. Indeed, this finding was supported both by increased and earlier deterioration of systolic function and by early elevation of markers of heart failure and remodelling (ANP, BNP and β -MHC) in hearts of mice treated with a combination of TRZ and DOX. Walker *et al.*²⁷ evaluated over 10 days mice receiving a combination of DOX (20 mg/kg) and TRZ (10 mg/kg). Although the mortality rate for DOX-treated mice was high (50%) at day 5, the mortality rate was even worse for DOX+TRZ-treated mice (80%). Jassal *et al.*¹⁴, using an acute murine model of chemotherapy-induced cardiotoxicity, observed that the mortality rate was extremely high (80%) in DOX+TRZ treated mice compared with that in mice with DOX treatment only. Mice treated with DOX or DOX+TRZ demonstrated progressive LV dilatation and reduced LVEF, compared with the group control, but systolic dysfunction appeared earlier in the group treated with DOX+TRZ.

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Conflict of interest: On behalf of all authors, the corresponding author states that there is no conflict of interest including financial and personal relationships with other people or organizations that could inappropriately influence this work.

The pharmacological target of TRZ in mice is still a matter of great debate. The potentiation of DOX-induced cardiomyopathy by TRZ could be explained by the impossibility of HER-2 survival pathways to overreact to the anthracycline-related stress in cardiomyocytes. Indeed, in mice, TRZ-induced cardiotoxicity has been attributed to the blockade of HER-2 signalling in cardiomyocytes, which lead to the alteration of antiapoptotic signalling pathways²⁷ and to an increase in oxidative stress. Gordon *et al.* showed that blockade of HER-2 enables the accumulation of reactive oxygen species (ROS) within neonatal rats' cardiomyocytes through a mitochondrial pathway, leading to the development of cardiac dysfunction by stimulating cardiomyocyte apoptosis¹⁶. In addition, increased angiotensin II levels as a result of HER-2 inhibition lead to an increase in ROS production and the inhibition of neuregulin signalling³⁴.

4. Conclusion

Our model of postnatal programming suggests, for the first time, an association between overweight and DOX-induced cardiotoxicity, resulting in greater sensitivity to the cardiotoxic effects of DOX, with significant acute systolic function impairment assessed by echocardiography. Furthermore, our results confirmed that TRZ administration potentiates the acute cardiotoxicity induced by DOX, especially in lean mice treated with DOX, but no significant additional effect was found in our model of moderately overweight mice.

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Tables

Table 1. Forward and reverse sequences of primers used for the amplification of mouse genes

	Forward (5' to 3')	Reverse (5' to 3')
<i>HPRT</i>	CTGGTGAAAAGGACCTCTCG	TGAAGTACTCATTATAGTCAAGGGCA
<i>ANP</i>	GAGAAGATGCCGGTAGAAGA	AAGCACTGCCGTCTCTCAGA
<i>BNP</i>	ATGGATCTCCTGAAGGTGCTG	GTGCTGCCTTGAGACCGAA
<i>β-MHC</i>	GCCAACACCAACCTGTCCAAGTTC	TGCAAAGGCTCCAGGTCTGAGGGC

Table 2. Left ventricular parameters

Variables	Baseline (Day 0)	Day 10	Day 20
HR (beats/min)			
NL CONT	392±51	398±40	409±38
NL DOX	390±47	388±51	407±56
NL TRZ	356±24	376±25	388±27
NL DOX-TRZ	354±56	370±42	373±40
SL CONT	388±14	391±27	432±116
SL DOX	402±49	395±56	398±57
SL TRZ	393±45	345±23	400±72
SL DOX-TRZ	356±39	362±40	375±22
LVEF (%)			
NL CONT	57±5	57±4	56±4
NL DOX	60±4	57±4	58±3
NL TRZ	58±5	54±7	52±2
NL DOX-TRZ	59±2	55±7	52±6†
SL CONT	56±3	56±3	55±3
SL DOX	61±5	49±7†*	47±4†*
SL TRZ	58±4	53±3	52±5
SL DOX-TRZ	58±3	49±4v*	48±5†
LVEDD (mm)			
NL CONT	4.2±0.3	4.2±0.2	4.3±0.3
NL DOX	4.0±0.2	4.0±0.2	4.0±0.3
NL TRZ	4.1±0.4	4.1±0.3	4.1±0.2
NL DOX-TRZ	4.0±0.2	4.0±0.2	4.0±0.1
SL CONT	4.3±0.2	4.2±0.2	4.2±0.2
SL DOX	4.1±0.3	4.4±0.3†*	4.3±0.2†*
SL TRZ	4.0±0.2	4.3±0.3	4.1±0.2
SL DOX-TRZ	4.2±0.3	4.4±0.3*	4.4±0.2*
LVMN (mg/g)			
NL CONT	4.3±0.5	4.3±0.8	4.2±0.9
NL DOX	3.9±0.7	3.8±0.7	3.8±0.8
NL TRZ	4.1±0.5	4.1±0.6	4.2±0.6
NL DOX-TRZ	4.0±0.3	4.0±0.4	4.3±0.3
SL CONT	4.0±0.3	3.9±0.3	4.0±0.3
SL DOX	3.7±0.7	4.0±0.9	3.9±0.7
SL TRZ	3.9±0.3	4.2±0.3	4.0±0.4
SL DOX-TRZ	4.2±0.4	4.5±0.8	4.4±0.3
E/A ratio			
NL CONT	1.4±0.1	1.4±0.2	1.4±0.2
NL DOX	1.4±0.1	1.5±0.2	1.3±0.2
NL TRZ	1.5±0.1	1.4±0.1	1.3±0.1
NL DOX-TRZ	1.6±0.2	1.6±0.6	1.5±0.5
SL CONT	1.4±0.3	1.4±0.2	1.5±0.6
SL DOX	1.5±0.2	1.4±0.2	1.4±0.2
SL TRZ	1.5±0.2	1.4±0.1	1.5±0.2
SL DOX-TRZ	1.5±0.2	1.4±0.1	1.3±0.1

Data are means ± standard deviation. DOX indicates doxorubicin; HR: heart rate; LVEDD: left ventricular end-diastolic diameter; LVEF: left ventricular ejection fraction; LVMN: left ventricular mass normalized to body weight; n: number of mice; NL: normal litter; SL: small litter. TRZ: trastuzumab. † $p < 0.01$ vs D0 in each group, * $p < 0.01$ compared with NL group with the same treatment, using 2-way ANOVA for repeated measures with a Tukey test as the post-hoc analysis

Figure Legends

Figure 1. Study protocol for the assessment of the cardiac effects of DOX, TRZ or their combination in 4-month-old control mice from a normal litter (NL) and overweight mice from a small litter (SL): Eight study groups were created, according to the single intra-peritoneal (IP) injection of DOX (DOX, 6mg/kg), TRZ (TRZ, 10mg/kg) or DOX+TRZ (DOX-TRZ) or saline at D1. At D0, D10 and D20 all mice underwent transthoracic echocardiography. A subset of mice was killed 48 hours after the injections (D3) and blood and tissue samples were harvested. At the end of the protocol, all mice were killed and blood and tissue samples were harvested.

Figure 2. Evolution of body weight in control mice from a normal litter (NL) and overweight mice from a small litter (SL) after a single intra-peritoneal (IP) injection at D1 of (A) saline, (B) DOX (DOX, 6mg/kg), (C) TRZ (TRZ, 10mg/kg) or (D) DOX+TRZ (DOX-TRZ).

* Different from NL group, $p < 0.01$

† Different from D0, $p < 0.05$

Figure 3. Evolution of left ventricular ejection fraction (LVEF) in control mice from a normal litter (NL) and overweight mice from a small litter (SL) after a single intra-peritoneal (IP) injection at D1 of (A) saline, (B) DOX (DOX, 6mg/kg), (C) TRZ (TRZ, 10mg/kg) or (D) DOX+TRZ (DOX-TRZ).

* Different from NL group, $p < 0.01$

† Different from D0, $p < 0.01$

Figure 4. Relative mRNA expression of (A) brain natriuretic peptide (BNP), (B) atrial natriuretic peptide (ANP) and (C) beta-myosin heavy chain (β -MHC) at D3 in control mice from a normal litter (NL) and overweight mice from a small litter (SL) after single intra-peritoneal (IP) injection of DOX (DOX, 6mg/kg), TRZ (TRZ, 10mg/kg) or DOX+TRZ (DOX-TRZ) or saline at D1.

Figure1

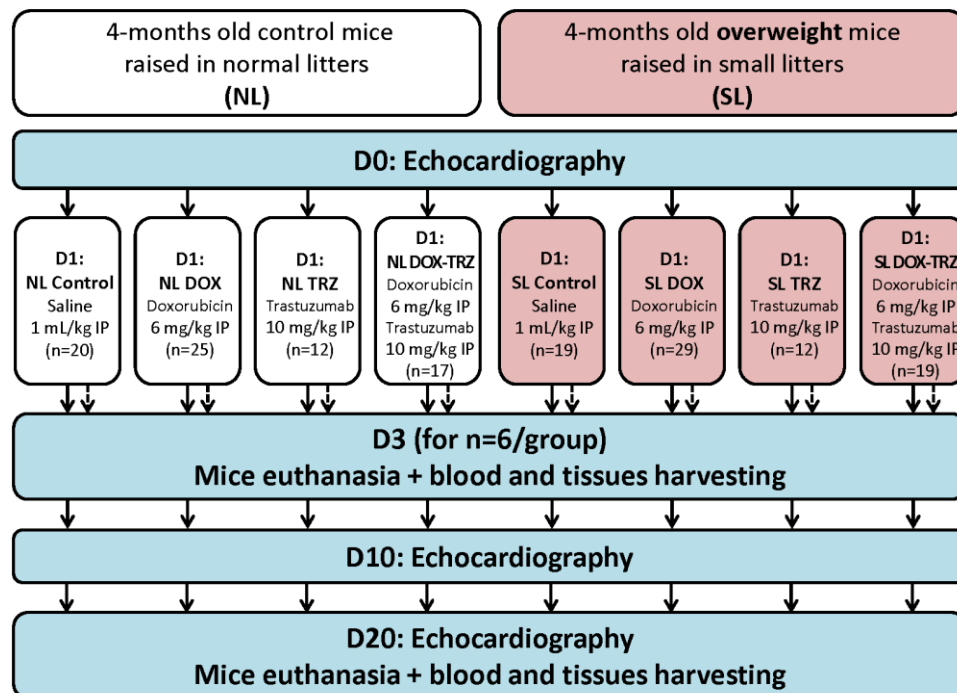


Figure2

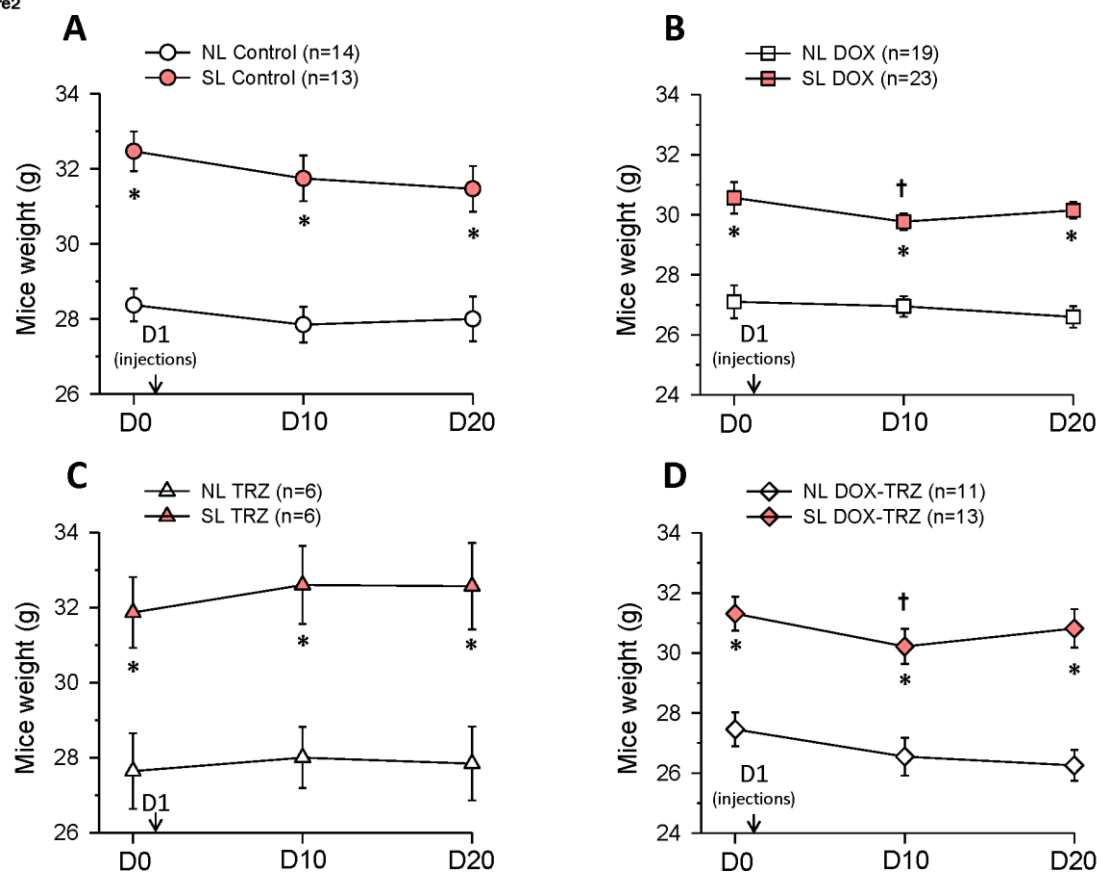


Figure3

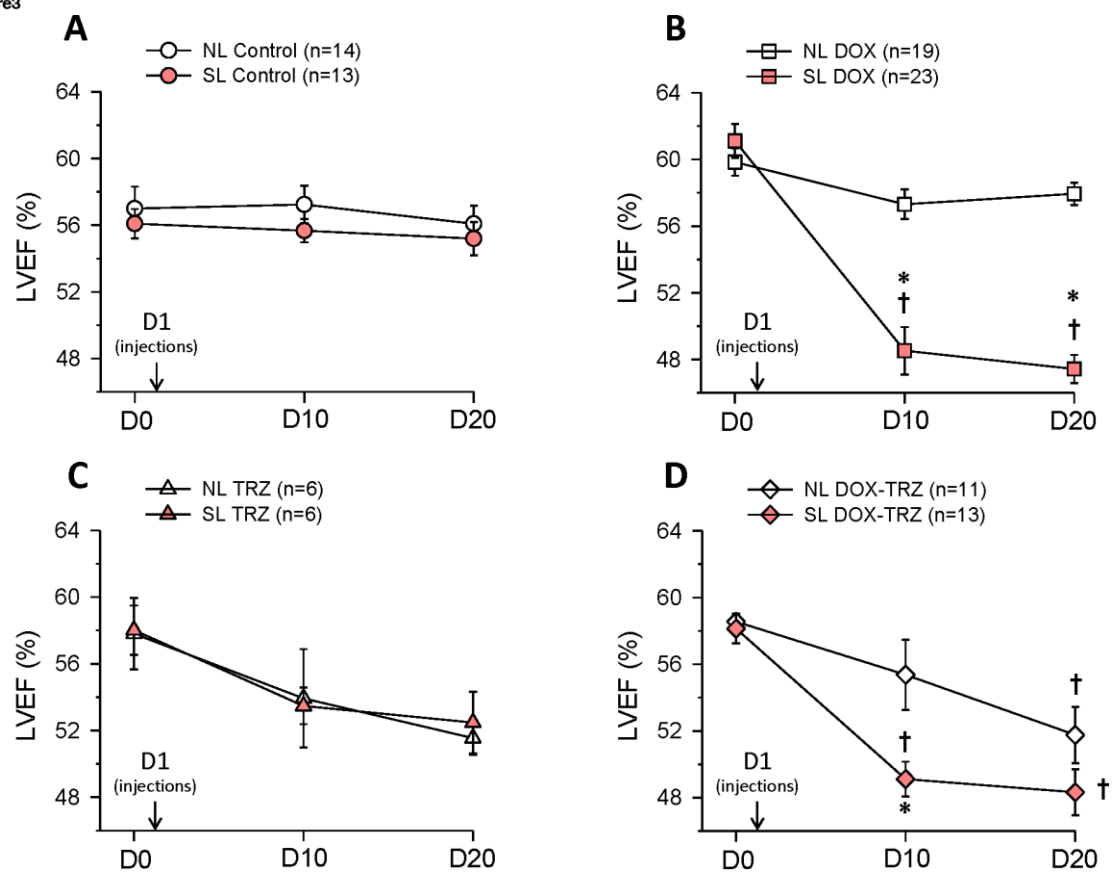
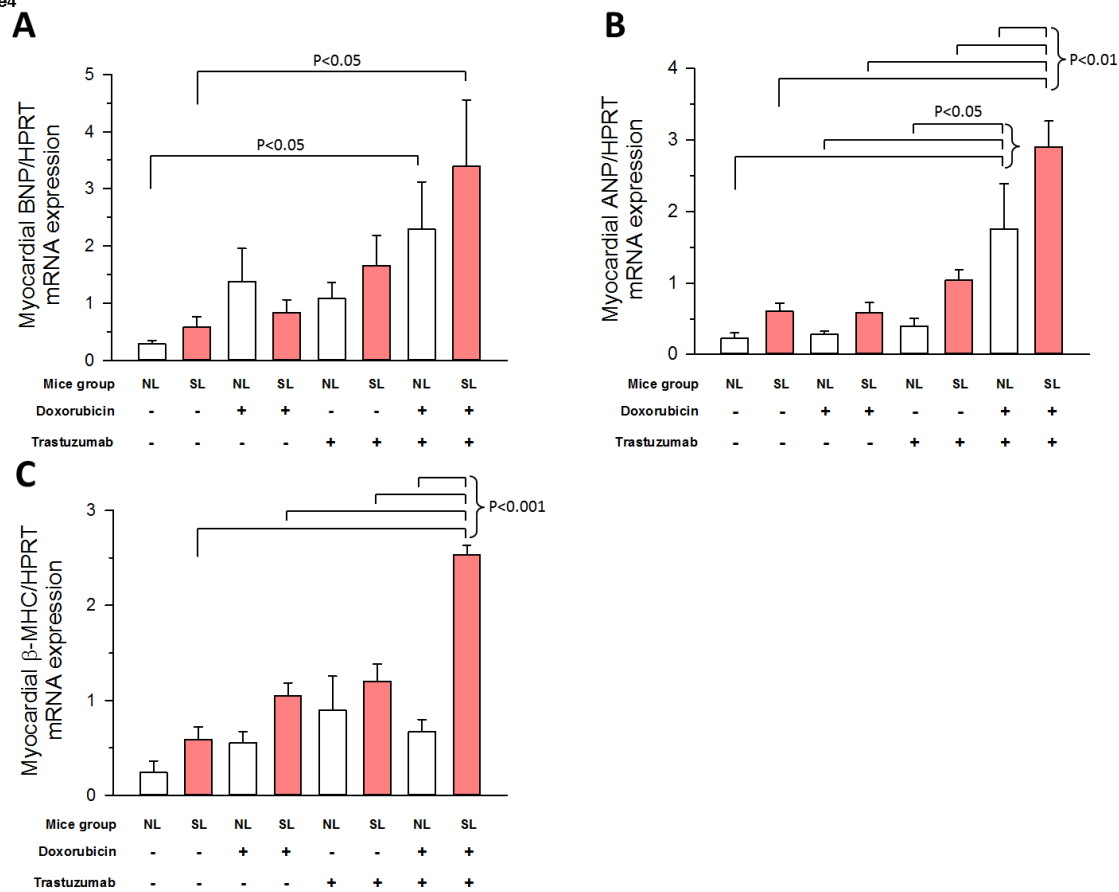


Figure4



Conclusion

Nous nous proposons dans cette conclusion de souligner les points essentiels des travaux présentés dans ce travail de thèse et d'envisager des perspectives de recherche.

A. Conclusion générale

Au travers de deux modèles murins, l'un portant sur la surcharge en fer et l'autre sur un surpoids induit précocement, nous avons mis en évidence le rôle majeur de déterminants métaboliques systémiques dans le développement de la cardiotoxicité aux chimiothérapies :

1. Les interactions entre le fer et les anthracyclines sont complexes : bien que le fer fournisse un substrat à la production d'espèces radicalaires par les anthracyclines, une surcharge en fer préalable à ce traitement semble protéger le myocarde contre les altérations fonctionnelles et tissulaires induites par la doxorubicine. Ce « préconditionnement » pourrait faire appel à la mise en jeu d'une augmentation des défenses anti-radicalaires par le fer. En effet, il a déjà été démontré qu'une surcharge en fer chronique est associée à une production accrue de radicaux libres, potentiellement délétère pour les cardiomyocytes (112). Dans notre étude expérimentale cependant, l'augmentation du stress oxydant cardiaque médiée par la surcharge en fer était associée à une majoration de l'expression myocardique des enzymes anti-oxydantes. Cependant, nous n'avons pas pu déterminer si cette régulation des enzymes anti-oxydantes était médiée directement par l'excès d'espèces radicalaires ou si des IRE (iron responsive elements) pourraient moduler post-transcriptionnellement l'expression génique de ces enzymes. De plus, le fer exerce un effet différent selon le type cellulaire : de fortes concentrations de fer-dextran protègent les cardiomyocytes H9c2 de la toxicité de la doxorubicine, alors qu'il majore celle-ci dans les lignées cellulaires cancéreuses EMT-6. Au sein même de la cellule, la localisation du fer semble déterminer son effet sur la cardiotoxicité : de récents travaux ont démontré qu'une surcharge mitochondriale en fer était associée à une incidence plus élevée de lésions cardiaques induites par les anthracyclines (91). Nos conclusions sont confortées par l'absence d'efficacité clinique retrouvée pour tous les chélateurs du fer actuellement disponibles, à l'exception du dexrazoxane dans des conditions bien spécifiques. Les bénéfices observés avec le dexrazoxane sur le plan de la cardioprotection contre les anthracyclines sembleraient plutôt liés à son activité catalytique d'inhibition de la topoisomérase II qu'à son effet chélateur du fer (36).

A l'aune de ces résultats, et des publications récentes sur le sujet, le modèle communément admis d'une potentialisation de la cardiotoxicité des anthracyclines par le fer doit être remis en question.

2. Dans un modèle de souris à risque cardiovasculaire accru, obtenu par suralimentation post natale, on observe une majoration de l'incidence et de la gravité de l'atteinte cardiaque induite par les anthracyclines comparé à des souris témoins. L'obésité est associée à un pronostic plus sombre chez les patientes traitées par anthracyclines pour un cancer du sein (103, 104), sans que les mécanismes impliqués dans ce sur-risque soient élucidés. La dérégulation des hormones issues des adipocytes, les adipocytokines, favorise le développement de pathologies liées à l'obésité. Ainsi, l'adiponectine plasmatique est plus basse chez les sujets obèses. Il a été démontré que l'adiponectine confère une moindre sensibilité cardiaque aux effets de la doxorubicine par le biais de l'activation de la voie Akt dans les cardiomyocytes. Les souris KO pour l'adiponectine ont une fonction ventriculaire gauche plus altérée que les souris contrôles après injection de doxorubicine. Ce sur-risque disparaît lorsque un apport exogène en adiponectine est fourni aux souris KO (106). Il est également prouvé que la metformine protège les cardiomyocytes des lésions cellulaires induites par les anthracyclines et que l'adiponectine cardiaque joue un rôle majeur dans cette action protectrice (107). Dans notre étude expérimentale, nous démontrons qu'un surpoids induit par programmation post-natale, modéré (poids des souris + 15% par rapport aux souris contrôles) est également associé à une plus grande susceptibilité à la toxicité cardiaque de la doxorubicine, à la fois sur le plan fonctionnel et moléculaire. Ces données démontrent l'importance des interactions entre le métabolisme systémique, et notamment du tissu graisseux, et les chimiothérapies cardiotoxiques utilisées dans le cancer du sein. Les mécanismes moléculaires impliqués dans cette potentialisation restent à élucider, et pourraient constituer des pistes thérapeutiques dans la prise en charge de la cardiotoxicité des anthracyclines.

Dans notre modèle, la cardiotoxicité du trastuzumab ne semble pas influencée par le surpoids, ce qui souligne la complexité et la diversité des mécanismes physiopathologiques impliqués dans la cardiotoxicité des différentes chimiothérapies. Cependant, il est possible que l'effectif de souris ait été insuffisant pour démontrer le sur-risque chez les souris en surpoids, du fait que l'atteinte cardiaque au trastuzumab est plus modérée que celle aux anthracyclines.

B. Perspectives

Au vu de nos résultats et de nos conclusions, deux axes de recherche complémentaires peuvent être envisagés, l'un qui s'oriente vers les mécanismes fondamentaux et l'autre à visée clinique.

1. Recherche fondamentale

La théorie mécanistique désormais admise pour la cardiopathie induite par les traitements anticancéreux est que la toxicité est plurifactorielle, impliquant à l'échelle cellulaire des mécanismes de croissance et d'hypertrophie, aboutissant à l'insuffisance cardiaque. De nouvelles voies de recherche pourraient être développées, basées sur une approche ciblée des mécanismes cellulaires impliqués.

Comme nous l'avons détaillé dans la synthèse bibliographique, des études récentes ont souligné les modifications induites par les anthracyclines sur les protéines régulatrices du fer (IRP) au sein des différents compartiments cellulaires, notamment dans la mitochondrie, entraînant l'accumulation de ce métal à haut potentiel oxydatif (91). Par ailleurs, il a été démontré que les anthracyclines diminuent précocement le potentiel de membrane, augmentent la concentration en calcium intra mitochondrial et modifient le potentiel redox de la mitochondrie (90). Les modifications du pH mitochondrial sont susceptibles de déclencher des mécanismes d'apoptose (113). Il a déjà été démontré *in vitro* que la réduction sélective du fer intra mitochondrial prévient l'apparition de la toxicité cardiaque des anthracyclines (91). Deux approches potentielles centrées sur le métabolisme semblent intéressantes pour la prophylaxie de la cardiotoxicité des anthracyclines chez l'humain : **le développement de nouveaux chélateurs du fer liant sélectivement (ou préférentiellement) le fer intra mitochondrial, et la mise au point d'agents pharmacologiques augmentant l'export mitochondrial du fer**, par exemple par le biais d'une surexpression d'ATP binding cassette (ABC) ABCB7 ou ABCB8 (31, 85).

Par ailleurs, le développement de molécules ciblant l'hépcidine, l'hormone participant à la régulation du métabolisme du fer, pourrait constituer une piste alternative dans la prévention des altérations oxydatives induites par les anthracyclines. En effet, ce peptide joue un rôle

majeur dans l'export du fer, y compris dans les cellules cardiaques, en liant la ferroportine et ainsi diminuant la sortie de ce métal hors du cardiomyocyte (114). Il a été montré récemment que dans diverses conditions physiopathologiques (myocardite, infarctus du myocarde, insuffisance cardiaque), l'expression de l'hepcidine est fortement augmentée au niveau du myocarde (115). Dans le cadre spécifique de la cardiotoxicité des anthracyclines et du trastuzumab, le rôle de l'hepcidine reste cependant à préciser.

En oncologie clinique, la prochaine décennie devrait assister le développement de nouvelles approches thérapeutiques de cardioprotection chez les patients traités par anthracyclines et trastuzumab (116). Comme nous venons de le préciser, l'homéostasie du fer dans les différents compartiments est essentielle aux fonctions cellulaires, mais jusqu'à présent les recherches pharmacologiques se sont avérées impuissantes à moduler finement et sélectivement les voies de régulations de ce métal qui restent complexes et interdépendantes. Dans ce contexte, l'avenir de la régénération pharmacologique pourrait se situer dans la délivrance localisée de molécules ciblant la croissance et la différenciation de voies spécifiques (117). Des travaux utilisant la parabiose chez la souris ont montré qu'un des membres de la famille des TGF- β , le GDF-11, serait capable d'inverser les mécanismes d'hypertrophie cardiaque observés avec le vieillissement (118, 119). Il s'agit d'une piste prometteuse sur la voie de la régénération myocardique, mais qui reste à valider chez l'humain. Au cours des dernières années, l'action bénéfique d'une autre cytokine nommée facteur inhibiteur de leucémie (LIF) a clairement été établie dans la prévention des altérations myocardiques associée à divers médicaments. LIF est un membre de la famille des cytokines de type IL-6, qui comprend l'IL-6, IL-11, IL-27, LIF, le facteur neurotrophique ciliaire (CNTF), cardiotropin-1 (CT-1), oncostatin M (OSM), et cardiotrophin-like cytokine-1 / novel neurotrophin-1 / B-cell stimulating factor-3 (CLC-1/NNT-1/BSF-3). Les données actuelles suggèrent que CT-1, une protéine de 201 acides aminés, joue également un rôle important dans la régulation du système cardio-vasculaire. CT-1 a un grand nombre de fonctions qui ont parfois des effets opposés. En fait, elle peut favoriser la survie des cellules cardiaques, mais peut également provoquer l'hypertrophie cardiaque et le remodelage ventriculaire (120). Pour LIF, le signal d'induction se produit lorsque cette cytokine se lie au récepteur LIF et déclenche sa dimérisation avec gp130. LIF active différentes voies dans les cardiomyocytes tels que (i) JAK-STAT et MAPK, et (ii) PI3K / Akt (121). Le mécanisme a été attribué à l'inhibition de l'ouverture du pore de transition de perméabilité mitochondriale en réponse à l'augmentation du Ca^{2+} intracellulaire et aux processus oxydatifs radicalaires (122). Les voies de

signalisation médiées par gp130 jouent un rôle important dans la progression de plusieurs types de cancer. L'inhibition de l'activité de la gp130 offre une approche et un potentiel prometteur pour le traitement de certaines formes tumorales.

2. Recherche clinique

Il est désormais clairement démontré que plusieurs thérapies médicamenteuses anti-cancéreuses, associées ou non à la radiothérapie, entraînent un risque accru d'atteinte myocardique (123). Idéalement, l'objectif des nouvelles thérapies devrait être d'améliorer la gestion du cancer à travers le ciblage spécifique des cellules malignes et la diminution des effets secondaires indésirables cardiaques. De nouvelles approches basées sur un traitement personnalisé du cancer émergent, impliquant le dépistage moléculaire des altérations génomiques cliniquement pertinentes et des thérapies ciblées sur le génotype (31). Les nouvelles thérapies comprennent des inhibiteurs de tyrosine kinase, des anticorps conjugués à la chimiothérapie, des inhibiteurs des heat shock proteins (HSP), et des anticorps qui interfèrent avec la formation des dimères ErbB2-ErbB3. De récentes analyses bioinformatiques et protéomiques ont mis en évidence plusieurs rôles, non constatés jusque là, pour des protéines spécifiques, dans la régulation de la physiologie cellulaire, en conditions normales et pathologiques. Le développement optimal et la mise en œuvre de thérapies ciblées sur HSP90 dépendra de la capacité à synthétiser les informations obtenues à partir d'une analyse génétique méticuleuse des tumeurs primaires et métastatiques. BIIB021 est le premier inhibiteur oral synthétique d'HSP90 qui a montré une efficacité clinique lorsqu'il est administré à des concentrations nanomolaires à des sujets porteurs de tumeurs solides avancées.

Les stratégies de prévention pharmacologique pourraient représenter un autre moyen de prévenir et/ou de réduire la cardiotoxicité. Certains biomarqueurs circulants cardiospécifiques constituent d'ores et déjà un outil validé pour la détection précoce et le suivi de la cardiotoxicité (124, 125)

La transition clinique des résultats obtenus dans notre première étude dans un modèle murin de surcharge en fer chronique peut être envisagée. Il serait alors intéressant de préciser chez des patients devant être traités par anthracyclines, si les taux circulants de fer, ferritine et hepcidine pourraient être associés au développement d'une cardiotoxicité. Est-ce qu'une

supplémentation en fer avant la chimiothérapie serait susceptible de prévenir certaines composantes fonctionnelles qui caractérisent la cardiotoxicité des thérapeutiques anticancéreuses ?

Une anamnèse détaillée des patients se focalisant les facteurs de risque cardiovasculaires et sur l'irradiation médiastinale est indispensable avant la mise en route d'un traitement potentiellement cardiotoxique. Au vu de nos résultats obtenus sur un modèle murin de surpoids induit précocement, le rôle du surpoids et de l'obésité dans l'initiation et le développement de la cardiotoxicité aux anthracyclines et au trastuzumab doit être évalué précisément chez l'humain. En effet, à l'heure actuelle, les données cliniques disponibles, la plupart du temps issues d'études de faible puissance, sont contradictoires et lacunaires quant au rôle du tissu adipeux sur l'incidence et la gravité de la cardiotoxicité des chimiothérapies anticancéreuses (125-127). Une méta-analyse systématique de la littérature permettrait dans un premier temps d'orienter les recherches, avant de mettre en place une large étude prospective multicentrique basée sur le rôle du métabolisme dans la cardiotoxicité de l'association anthracyclines-trastuzumab (évaluant des paramètres métaboliques physiologiques tels que le poids mais aussi le périmètre abdominal, la composition corporelle par bio-impédancemétrie, l'état nutritionnel, associés à des biomarqueurs circulants tels que l'adiponectine, l'hepcidine ou le GDF-11).

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Annexes

Liste des travaux :

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Communications orales

1. Congrès nationaux

Guenancia C, Li N, Habbout A, Cottin Y, Dutartre P, Garrido C, Rochette L, Vergely C. Iron overload does not potentiate doxorubicin induced cardiotoxicity in vivo in mice and in vitro in cardiomyocytes cell cultures. **Printemps de la Cardiologie 2014 Strasbourg**

Guenancia C, Chagué F, Cochet A, Loffroy R, Brunotte F, Cottin Y. Sudden cardiac death, beware of hasty diagnosis. **CFCI 2012 Paris** (*prix du meilleur cas clinique*)

2. Congrès internationaux

Guenancia C, Cochet A, Humbert O, Dygai-Cochet I, Lorgis L, Zeller M, Stamboul K, Brunotte F, Cottin Y. Ischemia but not necrosis is a predictor of post-stress LVEF drop 6 months after myocardial infarction: a gated myocardial perfusion SPECT study. **EANM 2012 Milan**

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Communications affichées

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Implications du stress oxydant et du fer dans la cardiotoxicité des anthracyclines et du trastuzumab

Le traitement du cancer a considérablement progressé ces dernières années, permettant une réduction de la mortalité. L'allongement de l'espérance de vie des patients a permis de mettre en évidence l'apparition retardée de toxicités cardiovasculaires induites par ces chimiothérapies. Les mécanismes physiopathologiques à l'origine de ces dysfonctions cardiaques sont complexes, intriqués et restent partiellement méconnus. Une meilleure compréhension des phénomènes impliqués dans ces cardiotoxicités est donc nécessaire afin de prévenir son apparition. En ce sens, nous avons développé deux approches expérimentales complémentaires visant à comprendre les mécanismes physiopathologiques impliqués dans la toxicité cardiaque des anthracyclines et du trastuzumab.

Une première étude expérimentale a permis de préciser le rôle du fer dans la cardiopathie induite aux anthracyclines. Nous avons démontré qu'une surcharge en fer chez la souris, préalable à l'injection de doxorubicine, ne majore pas la cardiotoxicité de cette chimiothérapie. Au contraire, la mise en jeu des défenses anti-radicalaires suite à la surcharge en fer pourrait limiter les lésions oxydatives cardiaques générées par la doxorubicine. A la vue de ces données, le rôle des chélateurs du fer dans la cardioprotection contre les anthracyclines est remis en question.

Notre deuxième travail expérimental visait à élucider le rôle du surpoids dans le développement de la cardiotoxicité des anthracyclines et du trastuzumab. Grâce à un modèle murin de surpoids modéré et de risque cardio-métabolique accru induit par programmation post-natale, nous avons mis en évidence le rôle potentiateur d'une surcharge pondérale sur le développement de la cardiotoxicité aux anthracyclines. La cardiotoxicité du trastuzumab ne semble pas être en revanche majorée par le surpoids. Nos travaux ont également permis de préciser les conditions dans lesquelles existent des potentialisations des effets lors de l'association doxorubicine et trastuzumab.

Mots clés

Cancer, cardiotoxicité, anthracyclines, trastuzumab, surpoids, stress oxydant.