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Novel organoselenium catalysts: From the heart of tuna fish to antioxidant capacity linked with nitric oxide signaling in cardiovascular diseases (SeleNOx)

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par **Rama ALHASAN**

Nouveaux composés organoséléniés : du cœur du thon aux capacités antioxydantes en passant par la signalisation complexe de monoxyde d'azote - seleNOx

16 avril 2024

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Abstract

Cardiovascular diseases are often linked to oxidative stress and reduced bioavailability of nitric oxide ($^{\bullet}NO$), leading to disruption of vascular homeostasis. Certain small, stable organoselenium molecules display antioxidant activity similar to that of glutathione peroxidase (GPx), as well as the ability to catalyse the release of $^{\bullet}NO$ from *S*-nitrosothiols (RSNOs). They can be of synthetic origin, such as ebselen, or natural, such as selenonein found in tuna. This study proposes the synthesis and evaluation *in tubo*, *in vitro* on human aortic smooth muscle cells (HuAoSMC) and *ex vivo* on isolated rat aortic rings, of new selenohydantoins inspired by selenonein. *In tubo* selenohydantoins **5e** (with -CF_3 substitution) and **5d** (with -CH_3 substitution) exhibited antioxidant, GPx-like activity and catalytic activity stimulating $^{\bullet}NO$ release from *S*-nitrosoglutathione (GSNO) greater than ebselen and selenocysteamine respectively. Selenohydantoins were shown to be cytocompatible with HuAoSMC, and **5e** proved to be the best catalyst for $^{\bullet}NO$ release from GSNO, significantly increasing the intracellular pool of $^{\bullet}NO$ formed from nitrite ions and *S*-nitrosothiols, without affecting cellular redox balance (GPx activity and intracellular glutathione concentration). Finally, **5e** and **5f** were shown to mobilize $^{\bullet}NO$ stored in the aorta in the form of *S*-nitrosothiols, inducing vasorelaxation of precontracted rat aortic rings. The results of this study suggest potential applications of synthesized selenohydantoins in the cardiovascular field, particularly in pathological conditions characterized by oxidative stress and impaired endothelial function.

Zusammenfassung

Kardiovaskuläre Erkrankungen sind mit oxidativem Stress und einer verminderten Bioverfügbarkeit von Stickstoffmonoxid ($^{\bullet}$NO) verbunden, was die vaskuläre Homöostase stört. Diese Studie untersucht die potentielle Modulation der vaskulären Funktion durch neue Organoselenmoleküle mit potentieller GPx-ähnlicher Aktivität, antioxidativer Aktivität und $^{\bullet}$NO-Freisetzung aus *S*-Nitrosothiolen (RSNOs). Diese Moleküle können synthetisch (wie Ebselen) oder natürlich (wie Selenonein in Thunfisch) sein. Inspiriert von Selenonein wurden neue Selenohydantoine synthetisiert und *in tubo*, *in vitro* an humanen glatten Muskelzellen der Aorta (HuAoSMC) und *ex vivo* an Ratten-Aortenringen evaluiert. Die Experimente *in tubo* zeigten eine interessante GPx-ähnliche Aktivität in der Nähe von Ebselen, insbesondere der Derivate **5e** (-CF_3) und **5d** (-CH_3). Selenohydantoine zeigten hohe antioxidative Aktivitäten und katalytische Eigenschaften, die die Freisetzung von $^{\bullet}$NO aus RSNOs stimulierten. Z.B. übertrafen die Derivate **5e** und **5f** Selenocystamin. *In vitro*- und *ex vivo*-Experimente zeigten die Zytokompatibilität der Selenohydantoine, wobei das Derivat **5e** die Protein-*S*-Nitrosation in HuAoSMC induzieren konnte, was den intrazellulären $^{\bullet}$NO-Pool signifikant erhöhte, ohne das zelluläre Redoxgleichgewicht (GPx-Aktivität und Glutathionkonzentration) zu beeinflussen. Wenn es mit *S*-Nitrosoglutathion (GSNO) coinkubiert wurde, ermöglichte es eine potentiell positive kardiovaskuläre Wirkung. Tatsächlich zeigten die Derivate **5e** und **5f** die Fähigkeit zur Vasorelaxation an vorverengten Ratten-Aortenringen und die Fähigkeit, GSNO-induzierte $^{\bullet}$NO-Reserven in der Aorta für die Vasorelaxation zu mobilisieren.

Résumé

Les maladies cardiovasculaires sont souvent liées au stress oxydant et à la réduction de la biodisponibilité du monoxyde d'azote (•NO), provoquant une perturbation de l'homéostasie vasculaire. Certains composés organoséléniés, stables et de petite taille, présentent une activité antioxydante similaire à celle de la glutathion peroxydase (GPx), ainsi qu'une capacité à catalyser la libération de •NO à partir des *S*-nitrosothiols (RSNOs). Ils peuvent être d'origine synthétique, comme l'ebselen, ou naturelle, comme la sélénonéine qui se trouve dans le thon. Cette étude propose la synthèse et évaluation *in tubo, in vitro* sur des cellules musculaires lisses aortiques humaines (HuAoSMC) et *ex vivo* sur des anneaux aortiques isolés de rat, de nouvelles sélénohydantoïnes inspirées par la sélénonéine. *In tubo* Les sélénohydantoïnes **5e** (-CF_3) et **5d** (-CH_3) ont présenté une activité GPx antioxydante et une activité catalytique stimulant la libération de •NO à partir du *S*-nitrosoglutathion (GSNO), respectivement plus importantes que celles de l'ebselen et de la sélénocystéamine. Les sélénohydantoïnes se sont montrées cytocompatibles avec des HuAoSMC et **5e** s'est révélé être le meilleur catalyseur de la libération de •NO à partir de GSNO en augmentant significativement le pool intracellulaire de •NO formé d'ions nitrites et de *S*-nitrosothiols, sans affecter l'équilibre redox cellulaire (activité GPx et concentration intracellulaire de glutathion). Enfin, **5e** et **5f** se sont révélés capables de mobiliser •NO stocké dans l'aorte sous forme de de *S*-nitrosothiols provoquant ainsi une vasorelaxation d'anneaux d'aorte de rat précontractés. Les résultats de cette étude suggèrent de potentielles applications de sélénohydantoïnes synthétisées dans le domaine cardiovasculaire, en particulier dans des conditions pathologiques caractérisées par un stress oxydant et une dégradation de la fonction endothéliale.

Introduction générale

Les maladies cardiovasculaires (MCV) notamment l'athérosclérose, l'hypertension et l'infarctus du myocarde représentent un problème de santé mondial et touchent le cœur et/ou les vaisseaux sanguins. La pathogenèse des MCV est souvent liée au stress oxydant, caractérisé par un déséquilibre entre la production d'espèces réactives de l'oxygène (ERO) et l'activité des défenses antioxydantes du corps. Ce déséquilibre peut provoquer des lésions cellulaires, une inflammation et une dégradation de la fonction vasculaire, contribuant ainsi au développement des maladies cardiovasculaires. Au centre de ces processus complexes se trouve l'endothélium, la fine couche de cellules qui tapisse la paroi interne des vaisseaux sanguins. L'endothélium joue un rôle essentiel dans la régulation du tonus vasculaire, le contrôle de l'inflammation et la prévention de la formation des caillots sanguins. Le monoxyde d'azote ($^{\bullet}NO$), un médiateur gazeux synthétisé par la $^{\bullet}NO$ synthase endothéliale (eNOS), est un élément essentiel de la fonction endothéliale. $^{\bullet}NO$ active la guanylate cyclase soluble (sGC), entraînant la formation de guanosine monophosphate cyclique (GMPc), un second messager qui déclenche des voies de signalisation en aval qui favorisent la vasodilatation, la cardioprotection et la réduction de l'inflammation. Cependant, la dérégulation de la production de $^{\bullet}NO$ peut contribuer au développement des MCV. Ainsi, la dysfonction endothéliale caractérisée par une production diminuée de $^{\bullet}NO$ et un découplage de la eNOS, apparaît comme une phase initiale et réversible de la progression des MCV. Ce dysfonctionnement mène à une vasodilatation insuffisante, à un stress oxydant élevé et à l'inflammation, ce qui aggrave encore le processus de développement des MCV. Les *S*-nitrosothiols (RSNO) formés par l'addition réversible de $^{\bullet}NO$ sur un groupement fonctionnel thiol, notamment celui des résidus cystéine des protéines, sont une forme de stockage et de transport de $^{\bullet}NO$ et jouent un rôle essentiel dans la régulation de nombreux mécanismes cellulaires, dont la migration, la perméabilité, le stress oxydant, le vieillissement et l'inflammation. Dans le contexte cardiovasculaire, les RSNO améliorent le tonus vasculaire, l'agrégation plaquettaire et la fonction des cellules immunitaires.

En raison de leurs diverses activités biologiques, notamment antioxydantes, anti-inflammatoires et cytoprotectrices, les composés organoséléniés sont des agents thérapeutiques intéressants dans le cadre des maladies cardiovasculaires. Ces activités biologiques proviennent des propriétés uniques d'oxydoréduction du sélénium, un oligo-élément essentiel qui joue un rôle fondamental dans divers processus physiologiques. Les composés organoséléniés peuvent être d'origine synthétique comme l'ebselen, ou d'origine naturelle, comme les sélénoprotéines (par exemple les glutathion peroxydases (GPx)) et la sélénonéine, une nouvelle molécule d'origine marine qui possède une forte capacité antioxydante. L'ebselen est le chef de fil des composés séléniés à activité GPx présentant également des propriétés anti-inflammatoires et cardioprotectrices potentielles. Cependant, la faible solubilité

aqueuse de l'ebselen limite ses applications thérapeutiques. Pour résoudre ce problème de solubilité, des efforts ont été faits pour synthétiser d'autres composés organoséléniés, en particulier des dérivés de la sélénohistidine et de la sélénohydantoïne. Les sélénohydantoïnes, inspirés de la molécule naturelle sélénonéine, possèdent une meilleure solubilité et des avantages thérapeutiques potentiels.

Ainsi, cette thèse a pour objectifs de :

1. Synthétiser une nouvelle série de sélénohydantoïnes, les caractériser et étudier leur activité antioxydante, leur activité GPx-like et leur capacité à catalyser la libération de •NO à partir de GSNO *in tubo*, et proposer des composés aux résultats intéressants.
2. Évaluer *in vitro* la cytocompatibilité des sélénohydantoïnes sur les cellules musculaires lisses aortiques humaines (HuAoSMC), et étudier les effets des molécules intéressantes sur la biodisponibilité de •NO pour les HuAoSMC.
3. Evaluer, à l'aide d'études *ex vivo* utilisant des anneaux aortiques de rats isolés, les effets vasodilatateurs directs et indirects (biodisponibilité de •NO) des sélénohydantoïnes sélectionnées.

Le positionnement de ce sujet de thèse par rapport à la littérature a donné lieu à la publication de trois articles de revues et les résultats obtenus ont donné lieu à deux articles originaux (un accepté et un soumis) tous résumés ci-après.

<u>Article I:</u> **Selenium Donors at the Junction of Inflammatory Diseases**

Les Donneurs de Sélénium à la Jonction des Maladies Inflammatoires.

Rama Alhasan, Ammar Kharma, Pierre Leroy, Claus Jacob and Caroline Gaucher

Current Pharmaceutical Design, 2019, 25: 1-10.

Le sélénium est un oligo-élément essentiel non métallique, dont des apports non adaptés sont liés à diverses maladies, notamment des affections graves telles que le syndrome de détresse respiratoire, l'infarctus du myocarde et l'insuffisance rénale (surcharge en sélénium), et des maladies inflammatoires chroniques telles que les maladies inflammatoires de l'intestin, la polyarthrite rhumatoïde et l'athérosclérose (déficit en sélénium). La principale source de sélénium provient du régime alimentaire, dérivé de sources animales et céréalières, et son absorption intestinale est limitée à la sélénocystéine et à la sélénométhionine et à leur incorporation dans les sélénoprotéines. Cette revue décrit (ou rapporte) entre le sélénium et les maladies inflammatoires et explore le potentiel des nanoparticules de sélénium et des composés organoséléniés pour compenser un déficit alimentaire en sélénium. Grâce à un taux de charge élevé en sélénium, les nanoparticules permettent, à faible dose, de restaurer la biodisponibilité du sélénium, alors que les composés organoséléniés jouent un rôle en modulant leurs activités antioxydantes ou anti-inflammatoires.

En effet, le sélénium joue un rôle essentiel dans la synthèse de sélénocystéine, un composant central de l'activité catalytique d'enzymes comme la glutathion peroxydase (GPx). De plus, le sélénium joue plusieurs rôles importants dans l'organisme, notamment par l'intermédiaire d'effets anti-mutagènes, anti-cancérigènes, antiviraux, antibactériens et antifongiques. Le sélénium est un élément important qui possède des activités antioxydantes et modulatrices redox qui peuvent également stimuler la synthèse ou la libération de monoxyde d'azote (•NO) à partir de *S*-nitrosothiols endogènes. Les composés organoséléniés ont la capacité de renforcer la *S*-nitrosation des protéines et la mobilisation de •NO dans les cellules aortiques, ce qui est particulièrement intéressant dans les maladies cardiovasculaires (MCV). Elles favorisent la vasodilatation, améliorent la fonction endothéliale et ont le potentiel de restaurer l'homéostasie vasculaire, de prévenir ou de traiter diverses pathologies cardiovasculaires. Ces composés, associés aux substances qui libèrent •NO, peuvent protéger les résidus de cystéine (Cys), et pourraient offrir collectivement un potentiel thérapeutique efficace pour les MCV et d'autres problèmes de santé.

La publication est disponible sur EurekaSelect *via* DOI: 10.2174/1381612825666190701153903

Article II: Selenoneine: A Unique Reactive Selenium Species from the Blood of Tuna with Implications for Human Diseases

Sélénonéine : Une Espèce Réactive du Sélénium Unique provenant du Sang du Thon avec des Applications potentielles dans les Maladies Humaines

Rama Alhasan & Muhammad Jawad Nasim & Claus Jacob & Caroline Gaucher

Current Pharmacology Reports, 2019, 5:163-173.

Objectif de l'étude : L'oligo-élément sélénium est présent dans de nombreux aliments, des céréales aux noix du Brésil. Chez l'Homme, ce chalcogène est essentiel pour de nombreux processus physiologiques. Il y a quelques années, la sélénonéine, un dérivé plutôt inhabituel de la séléno-histidine, a été isolé à partir du thon.

Résultats récents : Alors qu'il existe un nombre limité de petits composés de sélénium naturels, la sélénonéine peut être produite en grandes quantités dans des micro-organismes génétiquement modifiés et par synthèse chimique. En raison de la tautomérie rare du sélénol/sélénone, ce composé présente des propriétés d'oxydoréduction uniques et des activités biologiques intéressantes, qui vont de l'action antioxydante classique à l'interaction et à la protection subséquente des ions métalliques.

Résumé : la sélénonéine pourrait en effet constituer une première base prometteuse pour une nouvelle génération de suppléments de sélénium et d'agents chimiopréventifs.

La publication est disponible *via* DOI: 10.1007/s40495-019-00175-8

Article III: **Analytical Methods for the Quantification of Selenium Species in Biological Matrix: Where are We?**

Méthodes analytiques pour la quantification des espèces séléniées dans les matrices biologiques : Où en sommes-nous ?

Rama Alhasan, Caroline Perrin-Sarrado, Claus Jacob and Caroline Gaucher

Current Nutraceuticals, 2022, 3, 1:10.

Objectif : Au fil des ans, les recherches scientifiques ont démontré l'importance du sélénium en tant qu'élément essentiel pour les mammifères, soulignant son activité contre de nombreuses maladies et également ses effets prophylactiques. Il a également été établi qu'une consommation inadéquate de sélénium pouvait être délétère. Par conséquent, la nature et la concentration du sélénium et de ses dérivés présents dans l'alimentation, dans l'organisme et dans l'environnement, par exemple dans le sol, doivent pouvoir être déterminées avec soin.

Méthodes : Dans cette revue, les méthodes analytiques pour l'identification et la quantification de sélénium dans les échantillons biologiques sont résumées.

Résultats : Des méthodes utilisées en routine aux méthodes plus avancées sont explorées en se concentrant sur leurs caractéristiques analytiques, comme la spécificité des différents types de sélénium, la sensibilité, la précision, la reproductibilité et les compétences requises.

Conclusion : Il existe déjà de nombreuses études concernant l'analyse des espèces de sélénium. Au-delà de la méthode de quantification employée, nous proposons de passer en revue les étapes pré-analytiques de manipulation des échantillons biologiques qui affectent directement les résultats dont la précision est améliorée avec un prétraitement soigné. En outre, pour obtenir de meilleurs résultats en termes d'identification des espèces séléniées, différentes combinaisons de techniques peuvent s'avérer utiles. Nous soulignons ici les dernières méthodes de pointe pour identifier et quantifier le sélénium, telles que la chromatographie liquide à haute performance combinée à la spectrométrie de masse à plasma inductif (HPLC-ICP-MS), la spectrométrie d'absorption atomique à génération d'hydrure (HG-AAS), la spectrométrie de fluorescence atomique combinée à la génération d'hydrure (HG-AFS), ou la spectrométrie d'émission optique à plasma inductif (HG-ICP-OES). Cette étude souligne l'importance de ces recherches et la nécessité de parvenir à une quantification et à des méthodes de détermination fiables, sûres et efficaces.

La publication est disponible sur EurekaSelect *via* DOI: 10.2174/2665978602666211117154655

Article IV: **Selenoneine-inspired selenohydantoins with glutathione peroxidase-like activity**

Sélénohydantoïnes inspirées de la sélénonéine présentant une activité similaire à celle de la glutathion peroxydase

Rama Alhasan, Guilherme M. Martins, Pedro P. de Castro, Rahman Shah Zaib Saleem, Ali Zaiter, Isabelle Fries-Raeth, Alexandra Kleinclauss, Caroline Perrin-Sarrado, Patrick Chaimbault, Eufrˆanio N. da Silva Júnior, Caroline Gaucher, Claus Jacob

Bioorganic & Medicinal Chemistry, 2023, 94, 117479.

Plusieurs maladies chroniques, comme la mucoviscidose, certaines maladies inflammatoires de l'intestin, la polyarthrite rhumatoïde et certaines maladies cardiovasculaires, ont été liées à une diminution des taux de sélénium et à une augmentation du stress oxydant. Le sélénium est un oligo-élément essentiel qui présente des propriétés antioxydantes et les enzymes à sélénocystéines telles que la glutathion peroxydase sont particulièrement efficaces pour réduire les peroxydes. Dans cette étude, une série de composés organoséléniés ont été synthétisés et évalués pour leurs activités antioxydantes potentielles. Ces nouvelles molécules de sélénohydantoïne sont inspirées de la sélénonéine et synthétisées à partir de méthodes simples. Leur potentiel antioxydant a été évalué par plusieurs méthodes classiques de piégeage des radicaux libres et de réduction des métaux. Les dérivés de sélénohydantoïne ont montré une activité similaire à celle de la glutathion peroxydase, qui réduit les péroxydes comme le peroxyde d’hydrogène et les peroxydes organiques. Les calculs théoriques effectués à l'aide de la théorie fonctionnelle de la densité (TFD) ont montré que l'isomère sélénone était le seul présent en solution aqueuse, le sélénolate étant une forme tautomérique possible en présence d'une espèce basique. Les tests de cytocompatibilité ont indiqué que les dérivés de sélénohydantoïne ne sont pas toxiques pour les cellules musculaires lisses aortiques humaines primaires, ce qui ouvre la porte à des évaluations biologiques plus avancées de l'activité antioxydante de ces composés. Les résultats indiquent que les dérivés de la sélénohydantoïne portant des substituants trifluoro-méthyl (-CF_3) et chlore (-Cl) ont des activités importantes et pourraient être des candidats potentiels pour d'autres essais biologiques. Ces composés pourraient contribuer au développement de thérapies efficaces pour les maladies chroniques telles que les maladies cardiovasculaires.

La publication est disponible *via* https://doi.org/10.1016/j.bmc.2023.117479

Le fichier de données supplémentaires est disponible ici : https://ars-els-cdn-com.bases-doc.univ-lorraine.fr/content/image/1-s2.0-S0968089623003279-mmc1.doc

Article V: **Selenohydantoins Targeting Vascular Homeostasis by modulating Nitric Oxide Bioavailability and Vasorelaxation**

Les sélénohydantoïnes ciblent l'homéostasie vasculaire en modulant la biodisponibilité du monoxyde d'azote et la vasorelaxation

Rama Alhasan, Caroline Perrin-Sarrado, Isabelle Fries-Raeth, Claus Jacob, Caroline Gaucher

(à soumettre)

La régulation complexe de l'homéostasie vasculaire joue un rôle essentiel dans le maintien de la santé cardiovasculaire. Cette étude explore la modulation potentielle de la fonction vasculaire par l'introduction de nouvelles sélénohydantoïnes, une classe de composés avec une importance pharmacologique émergente. L'objectif était d'évaluer leurs effets sur la libération de monoxyde d'azote (•NO) à partir du *S*-nitrosoglutathion (GSNO) et la biodisponibilité de •NO pour les cellules musculaires lisses, ainsi que l'activité vasorelaxante subséquente sur les anneaux aortiques isolés.
L'étude a utilisé différentes méthodologies pour analyser l'interaction entre les deux composés intéressants qui possèdent une activité de type glutathion peroxydase (GPx), telles que **5e** avec une substitution trifluorométhyle (CF_3) et **5f** avec une substitution méthyle (CH_3), et le S-nitrosoglutathion (GSNO). Tout d'abord, nous avons montré *in tubo* que **5e** et **5f** étaient capables de libérer •NO à partir de GSNO avec une efficacité supérieure à celle de la sélénocystamine dans le cas de **5e**. Cette observation a été confirmée *in vitro*, où la co-incubation de **5e** avec GSNO a augmenté de manière significative la formation d'un pool intracellulaire de •NO sans affecter l'équilibre redox cellulaire (activité GPx et concentration intracellulaire de GSH), matérialisant la biodisponibilité de •NO et un potentiel effet protecteur cardiovasculaire. Par ailleurs, **5e** a également montré sa capacité *ex vivo* à mobiliser le pool de •NO généré par GSNO et à induire la relaxation d'aortes isolées de rat.

Cette étude montre les applications thérapeutiques prometteuses de ces sélénohydantoïnes, en particulier pour les conditions physiopathologiques caractérisées par une dégradation de la fonction endothéliale et propose des possibilités d'exploration plus avancées pour les applications physiologiques et pathologiques au sens large.

Discussion générale

Les maladies cardiovasculaires (MCV) restent un problème mondial de santé publique grave, qui demande une recherche continue de stratégies thérapeutiques innovantes pour lutter contre leur étiologie complexe [1,2]. D'une part, le stress oxydant, un déséquilibre entre la production d'espèces réactives de l'oxygène (ERO) et les mécanismes de défense antioxydante tels que la glutathion peroxydase (GPx), joue un rôle crucial dans l'initiation et la progression des MCV avec la diminution de la biodisponibilité de •NO et la dégradation des fonctions de l'endothélium. D'autre part, les antioxydants sont essentiels pour neutraliser les ERO et maintenir l'équilibre redox. En outre, les composés organoséléniés sont un exemple important d'antioxydants, leur rôle pouvant être attribué à leur potentiel de modulation de redox et à leur activité similaire à celle de la GPx. Les applications thérapeutiques potentielles des composés organoséléniés incluent la prévention des maladies, en particulier dans les conditions associées au stress oxydant, et leurs effets anti-inflammatoires renforcent encore leur importance. Cette étude a illustré le lien entre les capacités antioxydantes des composés organoséléniés et leur activité de libération de •NO à partir des réserves de •NO. Nos investigations se sont concentrées sur le potentiel des sélénohydantoïnes synthétisées récemment et inspirées de la sélénonéine [3–5].

La sélénonéine, isolée pour la première fois à partir du thon, est un composé unique de séléno-histidine comportant un atome de sélénium sur l'anneau imidazole. Ce composé organoséléné naturel présente une activité antioxydante significative, ce qui permet d'envisager un large spectre d'applications thérapeutiques. Cependant, en raison de sa nature hydrophile, son isolement à partir d'échantillons biologiques peut être difficile. De plus, sa synthèse chimique en plusieurs étapes est compliquée et ne donne que de très faibles rendements. Les dérivés de la sélénohydantoïne sont des exemples notables, partageant avec la sélénonéine une structure fondamentale d'anneau séléno-imidazole. Notre étude visait donc à faire la liaison entre les propriétés de la sélénonéine et les dérivés de la sélénohydantoïne, qui sont plus accessibles. Notamment, leur synthèse relativement simple avec de bons rendements les place au rang des molécules cibles importantes, en se concentrant sur leurs activités antioxydantes et de type GPx pour lutter contre le stress oxydant. En plus, les sélénohydantoïnes présentent une capacité à encourager la libération de •NO à partir des RSNO et ainsi à augmenter sa biodisponibilité concourant à améliorer et/ou suppléer à la fonction endothéliale et la santé cardiovasculaire en général [1,2,6–13]

Les composés sélénohydantoïnes synthétisés récemment, qui possèdent un atome de sélénium sur l'anneau imidazole, présentent une solubilité considérablement meilleure que celle de l'ebselen et une solubilité dans l'eau inférieure à celle de la sélénonéine, ce qui est important pour la manipulation et

la biodisponibilité. En outre, ils ont montré un spectre remarquable d'activités réductrices et de piégeage des radicaux. Notamment, leur efficacité dépasse celle des molécules antioxydantes de référence telles que le glutathion (GSH) et l'ebselen, le chef de file des mimes de GPx [3,14–18]. En particulier, le composé **5e** avec une substitution trifluorométhyle (-CF_3) a présenté une activité de type GPx comparable à celle de l'ebselen dans la réduction du peroxyde d'hydrogène (H_2O_2), et une activité de type GPx supérieure à celle de la sélénocystamine, qui est connue pour ses fortes propriétés antioxydantes [3,19]. En outre, le lien entre l'activité GPx et la libération de •NO a été un champ d'investigation scientifique. Par exemple, une carence en GPx3 est associée à une diminution de la biodisponibilité de •NO, ce qui suggère un lien potentiel entre l'activité de la GPx3 et la biodisponibilité de •NO [20]. De la même manière, une autre étude a discuté de la libération de •NO à partir des RSNO médiée par un mime de GPx, indiquant un lien entre l'activité de type GPx et la libération de •NO à partir des RSNO [21,22]. De nombreuses études dans la littérature ont déjà montré la capacité de différents composés organoséléniés à libérer •NO à partir des RSNO [23–27]. De plus, l'utilisation de composés organoséléniés dans la recherche cardiovasculaire est un domaine de recherche actif où ils ont pu être utilisés pour générer la libération de •NO en particulier quand ils peuvent imiter l'activité catalytique de la GPx, qui est capable de décomposer les RSNO, afin d'obtenir une libération de •NO stable et à long terme [28,29].

Ainsi, comme la diminution de la biodisponibilité de •NO est souvent liée au développement des MCV, nous avons étudié l'activité de libération de •NO par les sélénohydantoïnes à partir des RSNO, notamment GSNO, en présence de GSH comme agent réducteur, afin d'attester l'implication d'un cycle redox principalement lié à leur activité mimétique de GPx. Parmi les sélénohydantoïnes synthétisées récemment, les composés **5e** et **5f** ont tous deux présenté une activité de libération de •NO, l'activité de **5e** étant même supérieure à celle de la sélénocystamine, un composé organosélénié de référence qui a déjà été employée dans la libération de •NO à partir de GSNO [3,23–27,30–32]. De fait, l'introduction stratégique d'une substitution (-CF_3) au sein du composé **5e**, malgré sa nature non physiologique, a considérablement amélioré les activités de **5e** (*i.e.* mime GPx et libération de •NO). CF_3 est connu pour son effet attracteur d'électrons renforcé par les atomes de fluor directement attachés à l'atome de carbone produisant un effet inductif qui retire la densité électronique de l'anneau [3,33,34]. Ces différences viennent d'effets inductifs à longue portée sur le sélénium, un phénomène qui pourrait stabiliser l'intermédiaire séléno-sulfure au cours du cycle catalytique [35]. En outre, nos calculs théoriques ont également montré une distribution des formes sélénone/sélénolate en présence d'espèces basiques, ce qui permet la formation de sélénolate (RSe-) puisque les hydantoïnes ont un proton très acide qui prédomine sur la forme sélénol aux valeurs de pH physiologiques [3,36–38]. En outre, le sélénolate est très réactif et les actions antioxydantes directes des composés séléniés sont liées aux propriétés nucléophiles du sélénolate [3,5,23,39,40].

De plus, une étude récente a apporté la preuve de la formation d'espèces intermédiaires SeNO dans les mécanismes réactionnels de la génération séléno- catalysée de •NO à partir des RSNO et la formation rapide et concomitante d'un disélénide, en présence d'un thiol réducteur (GSH) [41]. Cependant, les sélénohydantoïnes ont montré moins d'activité vis-à-vis de *S*-nitroso-N-acetylcysteine (NACNO) et *S*-nitroso-N-acetylpenicillamine (SNAP) que vis-à-vis de GSNO. Cela suppose que le couplage du GSH avec sa propre forme nitrosée, le GSNO, pourrait être plus direct que le couplage avec NACNO ou SNAP. De plus, ces derniers composés pourraient potentiellement exercer un effet inhibiteur sur les composés ayant des activités de type GPx et des activités catalytiques [23,26,30–32]. Ainsi, diverses études ont démontré l'effet inhibiteur de SNAP sur l'activité de la GPx, probablement attribuable à la modification et à l'oxydation de résidus cruciaux tels que la cystéine ($Cys^{74 \text{ ou } 91}$) et la sélénocystéine ($Secys^{45}$) à l'intérieur de leurs sites actifs. Les groupes SeH du résidu Secys sont particulièrement impliqués dans ce processus, contribuant finalement à la désactivation de l'enzyme par la formation d'un pont sélényl-sulfure. Ce phénomène peut expliquer l'absence d'activité observée avec SNAP dans notre expérience *in tubo* [41–44].

Le potentiel important des sélénohydantoïnes, couplé avec leur cytocompatibilité vis-à-vis des HuAoSMC, les place au rang des candidats prometteurs pour une exploration plus approfondie en tant qu'antioxydant et joueur principal dans la catalyse de la formation et de la libération du pool de •NO. Notamment, les composés **5e** et **5f** sont ressortis en tête de liste et ont été choisis pour des investigations plus approfondies en raison de leur potentiel à contribuer à la formation du pool de •NO *in vitro* et à la libération du pool de •NO *ex vivo*. Les deux candidats ont montré leur capacité à libérer, à partir du GSNO, un •NO biodisponible pour les HuAoSMC matérialisé par une augmentation de la concentration intracellulaire en ions nitrites, la forme stable de •NO en milieux aqueux. Cependant, uniquement le composé **5e** a été capable d'augmenter la concentration intracellulaire de RSNO, suggérant qu'il existe une interaction synergique entre **5e** et GSNO pour induire la *S*-nitrosation et la libération catalytique de •NO. Cette observation est également en corrélation avec l'activité supérieure du **5e** dans la libération du •NO à partir du GSNO dans *in tubo* [25,30–32,45,46]. De plus, l'augmentation du pool de •NO intracellulaire indique une augmentation de •NO biodisponible, et sa signalisation par la protéine *S*-nitrosation, des aspects essentiels dans la régulation du tonus et de la fonction vasculaire [7,45,47–54].

Comme nous l'avons déjà discuté, le GSH joue un rôle central dans l'activité de la GPx et dans le cycle catalytique de libération de •NO à partir de GSNO. Nos expériences, ont montré des niveaux stables de GSH intracellulaire à travers les différentes conditions d'incubation *in vitro*, et en accord avec les niveaux élevés de GSH intracellulaire. L'activité antioxydante des composés **5e** et **5f** contribue à contrôler le stress oxydant, à maintenir l'homéostasie redox cellulaire et à protéger le pool de GSH,

parallèlement à la capacité de libérer un •NO biodisponible pour les cellules musculaires lisses sans perturber l'équilibre redox intracellulaire.

En outre, l'effet inhibiteur potentiel de •NO et de ses réserves sur l'activité de la GPx a déjà été discuté. Il a été montré que SNAP inactive la GPx, probablement par la modification SeNO et l'oxydation du résidu Sec et la formation d'un pont sélényl sulfide. Cet effet inhibiteur du SNAP sur la GPx est reversé par le dithiothréitol (DTT), ce qui suggère que la modification SeNO des sélénoenzymes peut être facilement réduite par des thiols [41–43,55–58]. Notre étude a montré que GSNO n'inhibait ni l'activité GPx de HuAoSMC ni l'activité GPx des composés **5e** et **5f** en lien avec la libération catalytique de •NO aboutissant à l'augmentation de son pool intracellulaire. Néanmoins, les conditions utilisées pour modifier les niveaux de •NO et la *S*-nitrosation n'ont pas perturbé l'homéostasie du GSH intracellulaire ou l'activité GPx, ce qui dévoile un mécanisme complexe. Cela suggère un double rôle potentiel dans le maintien de l'équilibre redox cellulaire tout en améliorant simultanément la biodisponibilité de •NO, ce qui pourrait cibler les voies de signalisation redox [59,60].

Enfin, afin d'utiliser les activités des sélénohydantoïnes pour améliorer la biodisponibilité de •NO et la santé vasculaire globale, nous avons étudié leur capacité à catalyser la libération de •NO à partir d'un pool de •NO stocké dans la paroi aortique *ex vivo* [48,61–67]. Nous avons d'abord montré que **5e** et **5f** présente une capacité vasorelaxante intrinsèque d'environ 30% à la fois sur des anneaux aortiques intacts ou sans endothélium et précontractés. Cela suggère que l'activité vasorelaxante des composés **5e** et **5f** est un phénomène indépendant de l'endothélium et qui n'est pas lié à la stimulation d'un récepteur ou d'une enzyme, car les courbes concentration-réponse ne correspondent pas à l'équation de Hill [18,26,66,68–71]. En outre, au regard des capacités de modulation des niveaux de •NO *in tubo* et *in vitro* déjà observées pour les composés **5e** et **5f**, d'autres expériences se sont avérées nécessaires pour évaluer ces activités *ex vivo*. [23,25–27,30]. Ainsi, les anneaux aortiques ont été soit pré-incubés avec GSNO pour former une réserve de •NO dans la paroi vasculaires, soit laissés non traités comme contrôles négatifs [53,63,66,72]. La NAC a été employée comme contrôle positif, puisque dans des études précédentes, elle a montré des activités vasorelaxantes uniquement *via* la mobilisation de •NO à partir du stock formé dans la paroi des artères [53,66,73–75]. De manière intéressante, en présence de **5e** et **5f**, les anneaux aortiques pré-incubés avec GSNO ont montré une vasorelaxation plus importante que ceux qui n'avaient pas de stockage de •NO. Ce qui suggère que **5e** et **5f** sont capables de libérer (dénitroser) •NO à partir des *S*-nitrosothiols/•NO formés par la pré-incubation avec GSNO avec les anneaux aortiques intacts et sans endothélium [53,63,66,72]. Les effets observés, suggérant un double mécanisme incluant des capacités antioxydantes et la libération de •NO, soutiennent leur impact potentiel sur la fonction vasculaire et le dysfonctionnement endothélial [11,61,69,74,76–78]. La capacité de ces composés à moduler la libération de •NO à partir des réserves vasculaires ajoute un niveau de

complexité, indiquant leur potentiel en tant que vasodilatateurs directs en mobilisant $^{\bullet}NO$ et en le redistribuant là où c'est nécessaire.

Finalement, les sélénohydantoïnes **5e** et **5f** synthétisées récemment, avec leur activité antioxydante et de libération de $^{\bullet}NO$, combinée à leur activité catalytique de type GPx, ouvrent une grande perspective en tant que candidats potentiels pour le traitement des MCV.

Conclusion et Perspectives

Cette étude a fourni une nouvelle série de sélénohydantoïnes prometteuses, une classe de composés organoséléniés inspirés par l'insaisissable sélénonéine, avec une solubilité et une stabilité améliorée et des impacts multiples sur le stress oxydant, la biodisponibilité de •NO et la fonction vasculaire. Le composé **5e**, en particulier, a émergé comme un candidat intéressant pour des interventions thérapeutiques dans le domaine de la santé cardiovasculaire et de la modulation redox.

Bien que notre étude présente des résultats prometteurs, la complexité qui se trouve derrière pourrait être limitante. L'examen de la stabilité à long terme des sélénohydantoïnes synthétisées récemment est un aspect qui demande de l'attention. De plus, ces résultats doivent être reproduits dans divers environnements de laboratoire et conditions expérimentales afin de garantir la robustesse et la reproductibilité de nos résultats au fil du temps et dans des circonstances variées.

De la même manière, il est impératif de comprendre la nature complexe de l'homéostasie redox des cellules et l'impact considérable des composés organoséléniées sur cette homéostasie. La recherche sur le sélénium est bien établie dans la biologie redox, en particulier dans le contexte des sélénoprotéines. Néanmoins, le potentiel d'activité redox du sélénium et sa susceptibilité à l'oxydation nécessitent un examen prudent de ses effets sur l'état redox des cellules. En outre, ses interactions avec les molécules contenant du soufre, y compris le GSH et les RSNO, doivent être évaluées avec précaution. Ces considérations sont essentielles pour faire progresser notre compréhension sur le rôle du sélénium dans la biologie redox et ses implications potentielles pour la santé. Alors que les expériences *in tubo* constituent sans aucun doute une base de recherche importante, la nature des expériences *in vitro* ou *ex vivo* présente une complexité supplémentaire en raison de la présence de différents composants et mécanismes cellulaires. Par conséquent, ces éléments ont un impact significatif sur les résultats. En outre, il est essentiel de reconnaître que l'absence de sites de liaison spécifiques *in vitro* ou *ex vivo* peut conduire à une sous-estimation du réel impact physiologique de ces sélénohydantoïnes, et diminuer les activités observées, comme on l'a vu avec l'activité GPx (*in tubo versus in vitro*). En outre, il est essentiel de surveiller les différents intermédiaires résultant des interactions avec le sélénium. Il s'agit notamment de suivre les différentes formes de sélénium (*i.e.* le sélénolate, le sélénol, la sélénone, le disélénide et l'acide sélénique), d'observer les intermédiaires sélénosulfures et d'identifier les espèces (SeNO) potentielles qui pourraient également contribuer à la libération catalytique de •NO. Comme nous l'avons déjà évoqué dans une précédente revue , de nombreuses méthodes spectroscopiques, telles que la spectroscopie UV-Vis et la résonance magnétique nucléaire (RMN), la spectrométrie de masse et la chromatographie liquide à haute performance (HPLC) sont en effet utiles pour détecter les différentes espèces de sélénium [79]. En plus,

les conditions expérimentales spécifiques employées, y compris des facteurs comme le temps d'incubation et les concentrations, méritent d'être examinées attentivement. Comme nous l'avons vu *in vitro*, des périodes d'incubation plus longues des composés **5e** et **5f** pourraient fournir des éléments supplémentaires. En effet, une interaction plus longue entre **5e** et GSNO modifie de manière significative le pool intracellulaire de $^{\bullet}$NO dans les HuAoSMCs. En outre, l'étude de différentes concentrations de sélénohydantoïnes pourrait également fournir une compréhension approfondie de leur relation concentration/activité. Une exploration plus poussée de l'interaction avec différents thiols est également nécessaire pour évaluer la capacité de ces sélénohydantoïnes à libérer $^{\bullet}$NO à partir de différents RSNO. En outre, les activités complexes observées *ex vivo* pourraient expliquer la réponse qui ne correspondent pas à l'équation de Hill, ce qui indique un manque de coopérativité directe dans la liaison de la molécule d'intérêt à un récepteur ou à une enzyme. Par conséquent, pour explorer l'implication de la voie sGC/cGMP, il est recommandé d'utiliser un inhibiteur spécifique de la sGC, tel que le H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), connu pour son inhibition puissante et sélective de l'activité GC stimulée par $^{\bullet}$NO. Parallèlement, l'étude de la modulation de différentes enzymes, notamment les nicotinamide adénine dinucléotide phosphate (NADPH) oxydases et la NO synthase endothéliale, est essentielle pour évaluer l'activité de vasorelaxation et le potentiel antioxydant. Notamment, les activités observées des composés **5e** et **5f** sur des anneaux aortiques sans endothélium suggèrent l'implication de mécanismes dépendants et indépendants de l'endothélium, ce qui est généralement évalué en utilisant des agents indépendants de l'endothélium comme contrôles positifs (par exemple, le nitroprussiate de sodium et la nitroglycérine) qui agissent directement sur les cellules musculaires lisses et provoquent une vasorelaxation. En complément, des études *in vivo* pourraient valider les résultats. En effet, l'étude des effets des composés dans des organismes vivants fournira une représentation plus réaliste de leur impact thérapeutique potentiel et aidera à diminuer le fossé entre la recherche en laboratoire et les applications cliniques potentielles. L'étude des effets à long terme est essentielle pour garantir leur potentiel en tant qu'agents thérapeutiques. Par exemple, des études de toxicité pour évaluer les effets indésirables des sélénohydantoïnes synthétisées sur des modèles animaux, afin d'évaluer la toxicité aiguë et chronique, telle que la mortalité, et tout signe de lésion d'un organe. De la même manière, il est essentiel de mener des études pour déterminer si une exposition prolongée aux composés organoséléniés peut entraîner l'apparition de tumeurs ou d'effets cancérigènes. En outre, il est tout aussi important d'évaluer leur stabilité métabolique (*i.e.* absorption, distribution, métabolisme, excrétion et interaction avec d'autres médicaments). Enfin, les composés organoséléniés ont déjà été utilisés pour la formulation de matériaux générateurs de $^{\bullet}$NO, en particulier les mimes de GPx, qui sont capables de décomposer les RSNO, et donc d'obtenir une libération de $^{\bullet}$NO stable et durable. De plus, le développement de matériaux pour une libération durable de $^{\bullet}$NO peut impliquer l'encapsulation ou

l'immobilisation de composés organoséléniés dans un vecteur adapté, tel qu'un polymère biocompatible ou d'autres matrices. Des techniques telles que la microencapsulation, l'incorporation dans des hydrogels ou la formulation de nanoparticules peuvent être explorées. En effet, la nanotechnologie peut être utilisée pour développer des formulations à base de nanoparticules pour l'administration de composés organoséléniés (*i.e.* sélénohydantoïnes), permettant potentiellement leur utilisation dans des méthodes de libération durable de •NO. Ces formulations peuvent améliorer la stabilité, la cinétique de libération contrôlée et la biocompatibilité des composés étudiés, facilitant ainsi leur application dans la recherche cardiovasculaire. Le nano-sélénium et ses applications en nano-médecine soulignent le potentiel de méthodes d'administration innovantes et d'amélioration de la biodisponibilité et de la sécurité.

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*Presentations **(PhD)**:*

<u>Oral communication:</u>

September, 2021 at The NutRedOx COST Action CA16112, Gdańsk, Poland**:** "*Novel organoselenium catalysts: From the heart of tuna fish to antioxidant capacity linked with nitric oxide signaling in cardiovascular diseases (SeleNOx)"*

<u>Poster presentations:</u>

June, 2023 at 25th International Redox Medicine Congress, Paris, France: "*Novel organoselenium catalysts: From the heart of tuna fish to antioxidant capacity linked with nitric oxide signaling in cardiovascular diseases (SeleNOx)"*

Doctoral school day, 2021, 2023: "Novel organoselenium catalysts: From the heart of tuna fish to antioxidant capacity linked with nitric oxide signaling in cardiovascular diseases (SeleNOx)"

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List of abbreviations

Cyclic guanosine 3′,5′-monophosphate: **cGMP**

Cysteine residues: **Cys**

Density functional theory: **DFT**

Dephosphorylates guanosine 5′-triphosphate: **GTP**

Disulfide isomerase: **PDI**

Endothelial nitric oxide synthase: **eNOS**

Experimental spectral peaks of absorbance Lambda max: **λmax**

Gamma-glutamyltransferase: **GGT**

Glutathione: **GSH**

Glutathione disulfide: **GSSG**

Glutathione peroxidases: **GPx**

Glutathione reductase: **GR**

Glutathione sulfinamide: **$GSONH_2$**

Glutathione sulfonic acid: **GSSOH**

Glutathione: **GSH**

High-resolution mass spectrometry: **HRMS**

Hydrogen peroxide: **H_2O_2**

Hydroxylamine: **NH_2OH**

Hydroxyl radical: **$^{\bullet}OH$**

Human aortic smooth muscle cells: **HuAoSMCs**

Mitogen-activated protein kinase: **MAPK**

Nicotinamide adenine dinucleotide hydrogen: **NADH**

Nicotinamide adenine dinucleotide phosphate: **$NADP^+$**

Nicotinamide adenine dinucleotide phosphate hydrogen: **NADPH**

Nicotinamide adenine dinucleotide hydrogen: **NAD^+**

Nitrate ion: **NO_3^-**

Nitric oxide: **$^{\bullet}NO$**

Nitric oxide synthase: **NOS**

Nitroxyl: **HNO**

N-hydroxysulfinamide: **GSNHOH**

Nitroso group: **R-NO**

Nuclear magnetic resonance: **NMR spectroscopy**

Peroxiredoxins: **Prx**

Peroxynitrite ion: **$ONOO^-$**

Peroxynitrous acid: **ONOOH**

Phosphodiesterases: **PDE**

Protein kinase G: **PKG**

Reactive oxygen species: **ROS**

Selenocysteine: **Secys**

S-nitroso-N-acetylcysteine: **NACNO**

S-nitroso-N-acetylpenicillamine: **SNAP**

S-nitrosoalbumine: **Alb-NO**

S-nitrosohemoglobin: **SNOHb**

S-nitrosothiols: **RSNO**

Soluble guanylate cyclase: **sGC**

Superoxide anion: **$O_2^{\bullet -}$**

Superoxide dismutase: **SOD**

Cysteine : **Cys**

Thioredoxin: **Trx**

Trx reductase: **TrxR**

Ultraviolet-visible: **UV-Vis**

General introduction

Cardiovascular disease (CVD), particularly atherosclerosis, hypertension and myocardial infarction, is a global health problem involving the heart and/or blood vessels. The pathogenesis of CVD is often linked to oxidative stress, which is characterised by an imbalance between the production of reactive oxygen species (ROS) and the activity of the body's antioxidant defences. This imbalance can lead to cell damage, inflammation and impaired vascular function, contributing to the development of cardiovascular disease. At the centre of these complex processes is the endothelium, the thin layer of cells that lines the inner lining of blood vessels. The endothelium plays an essential role in regulating vascular tone, controlling inflammation and preventing the formation of blood clots. Nitric oxide (•NO), a gaseous mediator synthesised by endothelial •NO synthase (eNOS), is an essential component of endothelial function. •NO activates soluble guanylate cyclase (sGC), leading to the formation of cyclic guanosine monophosphate (cGMP), a second messenger that triggers downstream signalling pathways that promote vasodilation, cardio-protection and reduced inflammation. However, deregulation of •NO production may contribute to the development of CVD. Thus, endothelial dysfunction, characterised by reduced •NO production and uncoupling of eNOS, appears to be an initial and reversible phase of CVD progression. This dysfunction leads to inadequate vasodilation, increased oxidative stress and inflammation, further exacerbating the process of CVD development. *S*-nitrosothiols (RSNOs), formed by the reversible addition of •NO to a thiol functional group, particularly that of protein cysteine residues, are a form of •NO storage and transport and play a key role in the regulation of many cellular mechanisms, including migration, permeability, oxidative stress, ageing and inflammation. In the cardiovascular context, RSNOs improve vascular tone, platelet aggregation and immune cell function.

Organoselenium molecules are attractive therapeutic agents for cardiovascular diseases due to their diverse biological activities, including antioxidant, anti-inflammatory and cytoprotective properties. These biological activities are due to the unique redox properties of selenium, an essential trace element that plays a fundamental role in various physiological processes. Organoselenium molecules can be of synthetic origin, such as ebselen, or of natural origin, such as selenoproteins (*e.g.* glutathione peroxidases (GPx)) and selenonein, a new molecule of marine origin with strong antioxidant capacity. Ebselen is the leading GPx-active selenium containing molecule with potential anti-inflammatory and cardioprotective properties. However, the low aqueous solubility of ebselen limits its therapeutic applications. To overcome this solubility problem, efforts have been made to synthesise other organoselenated molecules, in particular selenohistidine and selenohydantoin derivatives.

Selenohydantoins, inspired by the natural molecule selenoneine, have improved solubility and potential therapeutic advantages.

Therefore, this work aims to:

1. Synthesise a new series of selenohydantoins, characterise them and study their antioxidant activity, GPx-like activity and ability to catalyse the release of •NO from GSNO *in tubo*, and propose molecules with interesting results.
2. To evaluate in vitro the cytocompatibility of selenohydantoins on human aortic smooth muscle cells (HuAoSMC) and to study the effects of the molecules of interest on the bioavailability of •NO to HuAoSMC.
3. Evaluate the direct and indirect vasodilatory effects (bioavailability of •NO) of the selected selenohydantoins using *ex vivo* studies on isolated rat aortic rings.

The positioning of this thesis topic in relation to the literature led to the publication of three review articles and the results obtained led to two original papers (one accepted and one to be submitted), all of which are summarised below.

Allgemeine Einleitung

Kardiovaskuläre Erkrankungen (KHK) stellen weltweit ein großes Gesundheitsproblem dar und umfassen verschiedene von Erkrankungen des Herzens und der Blutgefäße, darunter Atherosklerose, Bluthochdruck und Herzinfarkt. Die Pathogenese der KHK ist eng mit oxidativem Stress verbunden, der durch ein Ungleichgewicht zwischen der Produktion reaktiver Sauerstoffspezies (ROS) und den antioxidativen Abwehrmechanismen des Körpers gekennzeichnet ist. Dieses Ungleichgewicht führt zu Zellschäden, Entzündungen und einer gestörten Gefäßfunktion, die zur Entwicklung der KHK beitragen.

Das Endothel steht im Mittelpunkt dieses komplizierten Zusammenspiels, die dünne Zellschicht, die die Innenwände der Blutgefäße auskleidet. Das Endothel spielt eine entscheidende Rolle bei der Regulierung der Gefäßspannung, der Kontrolle von Entzündungen und der Verhinderung von Blutgerinnseln. Ein wichtiger Faktor für die Endothelfunktion ist Stickstoffmonoxid ($^{\bullet}$NO), ein gasförmiges Signalmolekül, das von der Stickstoffmonoxidsynthase (eNOS) synthetisiert wird. $^{\bullet}$NO aktiviert die lösliche Guanylatcyclase (sGC), was zur Bildung von zyklischem Guanosinmonophosphat (cGMP) führt, einem zweiten Botenstoff, der nachgeschaltete Signalwege auslöst, die Vasodilatation, Kardioprotektion und Entzündungshemmung fördern.

Eine Fehlregulation der $^{\bullet}$NO-Produktion kann jedoch zur Entwicklung einer KHK beitragen. Die endotheliale Dysfunktion, die durch eine verminderte $^{\bullet}$NO-Produktion und eine Entkopplung der eNOS gekennzeichnet ist, tritt als frühe und reversible Phase im Verlauf der KHK auf. Diese Dysfunktion führt zu eingeschränkter Vasodilatation, erhöhtem oxidativen Stress und verstärkter Entzündung, was den KHK-Prozess weiter verschlimmert. *S*-Nitrosothiole (RSNO) sind ein weiterer wichtiger Bestandteil des Redox-Signalnetzwerks. Diese Moleküle entstehen durch die reversible Reaktion von $^{\bullet}$NO mit Thiolgruppen, wie sie an den Cysteinresten von Proteinen vorkommen. RSNOs spielen eine entscheidende Rolle bei der Regulation verschiedener zellulärer Prozesse wie Migration, Permeabilität, oxidativer Stress, Alterung und Entzündung. Im Zusammenhang mit der kardiovaskulären Gesundheit beeinflussen RSNOs die Gefäßspannung, die Thrombozytenaggregation und die Funktion von Immunzellen.

Organoselenmoleküle haben sich aufgrund ihrer vielfältigen biologischen Eigenschaften, darunter antioxidative, entzündungshemmende und zytotoxische Aktivitäten, als vielversprechende therapeutische Wirkstoffe für die Behandlung von KHK erwiesen. Diese Eigenschaften beruhen auf den einzigartigen Redoxeigenschaften von Selen, einem essentiellen Spurenelement, das in vielen physiologischen Prozessen eine wichtige Rolle spielt und sowohl synthetisch als auch natürlich in Form von Selenproteinen (z.B. Glutathionperoxidasen (GPx)) und Selenonein, einer neuartigen marinen Verbindung mit starker antioxidativer Kapazität, vorkommen kann.

Ebselen ist ein gut charakterisierter Vertreter der GPx-Mimetika mit potenziellen entzündungshemmenden und kardioprotektiven Wirkungen. Die schlechte Wasserlöslichkeit von Ebselen schränkt jedoch seine therapeutische Anwendung ein.

Um die Löslichkeitsprobleme zu lösen, wurden Anstrengungen unternommen, alternative Organoselenmoleküle zu synthetisieren, insbesondere Selenohistin- und Selenohydantoinderivate. Selenohydantoine, die von der natürlichen Verbindung Selenonein inspiriert sind, bieten eine verbesserte Löslichkeit und potenzielle therapeutische Vorteile.

Ziel dieser Arbeit:

1. Synthese einer neuen Reihe von Selenohydantoinen, Charakterisierung und *in tubo* Untersuchung ihrer antioxidativen Aktivität, Bewertung ihrer GPx-ähnlichen und katalytischen Aktivität mit Schwerpunkt auf ihrer potenziellen Fähigkeit, $^{\bullet}$NO aus GSNO freizusetzen, wobei bestimmte Derivate mit vielversprechenden Ergebnissen hervorgehoben werden.
2. *In vitro* Bewertung der Zytotoxizität von Selenohydantoinen auf humanen glatten Muskelzellen der Aorta (HuAoSMC) und Untersuchung der modulierenden Effekte vielversprechender Moleküle auf die Bioverfügbarkeit von $^{\bullet}$NO in HuAoSMC.
3. *Ex vivo*-Studien an isolierten Ratten-Aortenringen zur Bewertung der vasodilatatorischen Effekte und der Verbesserung der $^{\bullet}$NO-Bioverfügbarkeit durch die ausgewählten Selenohydantoine.

Die Positionierung dieses Dissertationsthemas in der Literatur führte zur Veröffentlichung von drei Zeitschriftenartikeln und die erzielten Ergebnisse führten zu zwei Originalarbeiten (eine akzeptiert und eine in Vorbereitung), die im Folgenden zusammengefasst

1 Chapter I: Introduction

1.1 The battle between the cardiovascular system and reactive species

Cardiovascular diseases (CVDs) are a broad term encompassing a range of conditions that affect the heart and blood vessels, such as coronary artery disease, myocardial infarction, stroke, heart failure, and peripheral arterial disease. CVDs are the leading cause of morbidity and mortality worldwide with many cases linked to oxidative stress, manifested as an imbalance between the production of reactive oxygen species (ROS) and antioxidants. ROS such as superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\bullet}OH$), are highly reactive molecules generated during physiological cell metabolism. At the cardiovascular level, the main sources of ROS are the mitochondrial electron transport chain, the activity of several redox enzymes such as cyclooxygenases, myeloperoxidases, lipoxygenases, xanthine oxidase, nicotinamide adenine dinucleotide phosphate hydrogen (NADPH), cytochrome P450 monooxygenase, uncoupled nitric oxide synthase (NOS) and peroxidases [80]. The fast diffusion-controlled reaction of nitric oxide ($^{\bullet}NO$) with $O_2^{\bullet-}$ forms the much more potent oxidant peroxynitrite ion ($ONOO^-$), which is protonated to peroxynitrous acid (ONOOH) after it captures a proton (H^+), ONOOH rearranges to HNO_3 which deprotonates again to H^+ and nitrate ion (NO_3^-). ROS formation starts at dioxygen (O_2) by capturing one electron, leading to the formation of $O_2^{\bullet-}$, which is the most abundant ROS in cells and is directly linked to the formation of all other forms of ROS, such as hydroxyl ($^{\bullet}OH$) radicals, hydroperoxyl ($^{\bullet}HO_2$) radicals and other non-radical species, such as H_2O_2 [81]. $O_2^{\bullet-}$ can be converted to H_2O_2 by the action of the superoxide dismutase (SOD) enzyme family. $O_2^{\bullet-}$ can also interact with hydrogen peroxide (H_2O_2) according to the Haber–Weiss reaction, leading to the production of $^{\bullet}OH$ [82]. H_2O_2 is involved in the production of $^{\bullet}OH$ according to the Fenton reaction in the presence of ferrous (Fe^{2+}) ions. Its presence may also result in the formation of hypochlorous acid (HOCl) by the action of myeloperoxidases in the presence of chloride ions (Cl^-). Notably, HOCl can interact further with H_2O_2 to form singlet oxygen ($^1O_2$) (Figure 1). Then again, H_2O_2 is reduced and thus detoxified by several enzymes: glutathione peroxidases (GPx), catalases, and peroxiredoxins (Prx). Indeed, ROS production needs to be balanced carefully because, when it exceeds the capacity of the mammalian antioxidant defence systems, it results in oxidative stress, which has detrimental effects on various biomolecules, including lipids, proteins, and DNA. These destructive interactions can lead to cell damage and dysfunction.

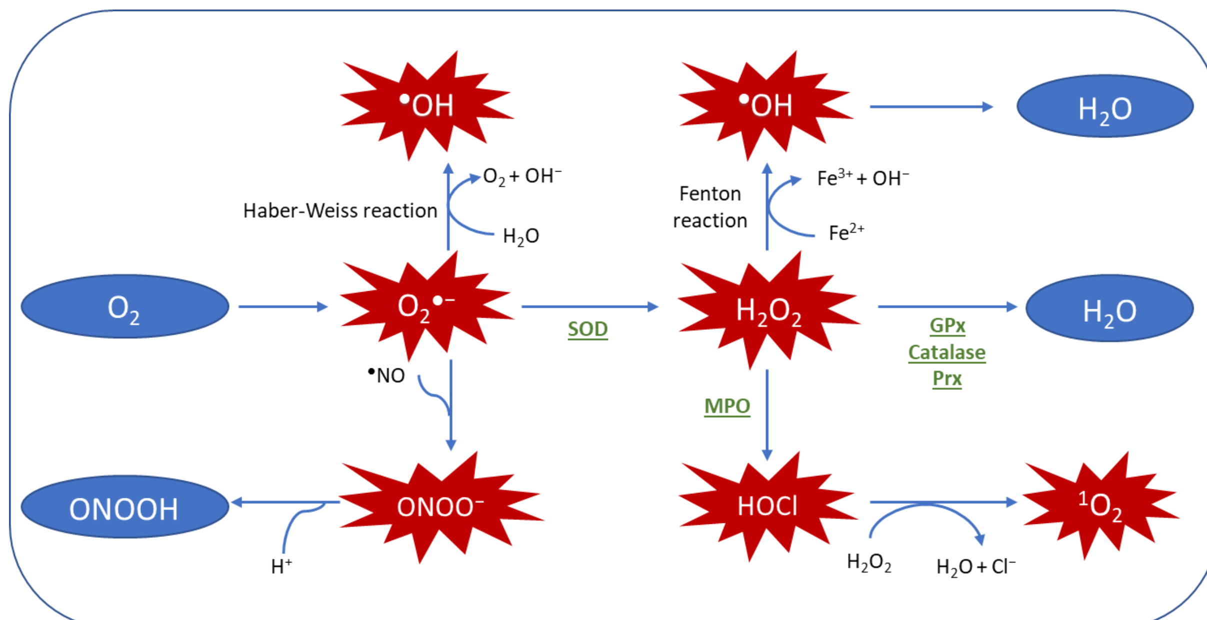

***Figure 1**: ROS formation and detoxification. $^1O_2$: singlet oxygen; Cl^-: chloride ion; Fe^{2+}: iron (II) ion; Fe^{3+}: iron (III) ion; GPx: glutathione peroxidase; H^+: proton; H_2O: water; H_2O_2: hydrogen peroxide; HOCl: hypochlorous acid; MPO: myeloperoxidases; •NO: nitric oxide; O_2: dioxygen; $O_2^{\bullet -}$: superoxide anion; •OH: hydroxyl radical; ONOO-: peroxynitrite; ONOOH: peroxynitrous acid; Prx: peroxiredoxins; SOD: superoxide dismutases.*

Within the cardiovascular system (Figure 2), oxidative stress creates an environment that contributes to the development of atherosclerosis damaging the inner lining of blood vessels (endothelium) by lipid accumulation in the arterial walls and triggering inflammation. This process leads to the formation of plaques that can narrow and block blood vessels, increasing the risk of heart attacks and stroke. Oxidative stress can also disrupt the delicate balance of calcium ions in cardiac muscle cells, potentially leading to irregular heartbeats or arrhythmias. It also plays a role in cardiac remodelling, which is the process by which the heart undergoes structural changes in response to stress or injury. Oxidative stress can activate hypertrophic signalling pathways, causing heart muscle thickening (hypertrophy), cardiomyocyte apoptosis, which can weaken the heart's pumping ability. Oxidative stress has also adverse effects on vascular homeostasis by reducing the availability of •NO, a molecule that helps to balance blood vessel dilation and constriction. When •NO levels decrease due to oxidative stress, which disrupts vascular homeostasis and leads to vasoconstriction, increasing the risk of platelet aggregation and immune cells recruitment narrowing blood vessels and increasing blood pressure [83,84].

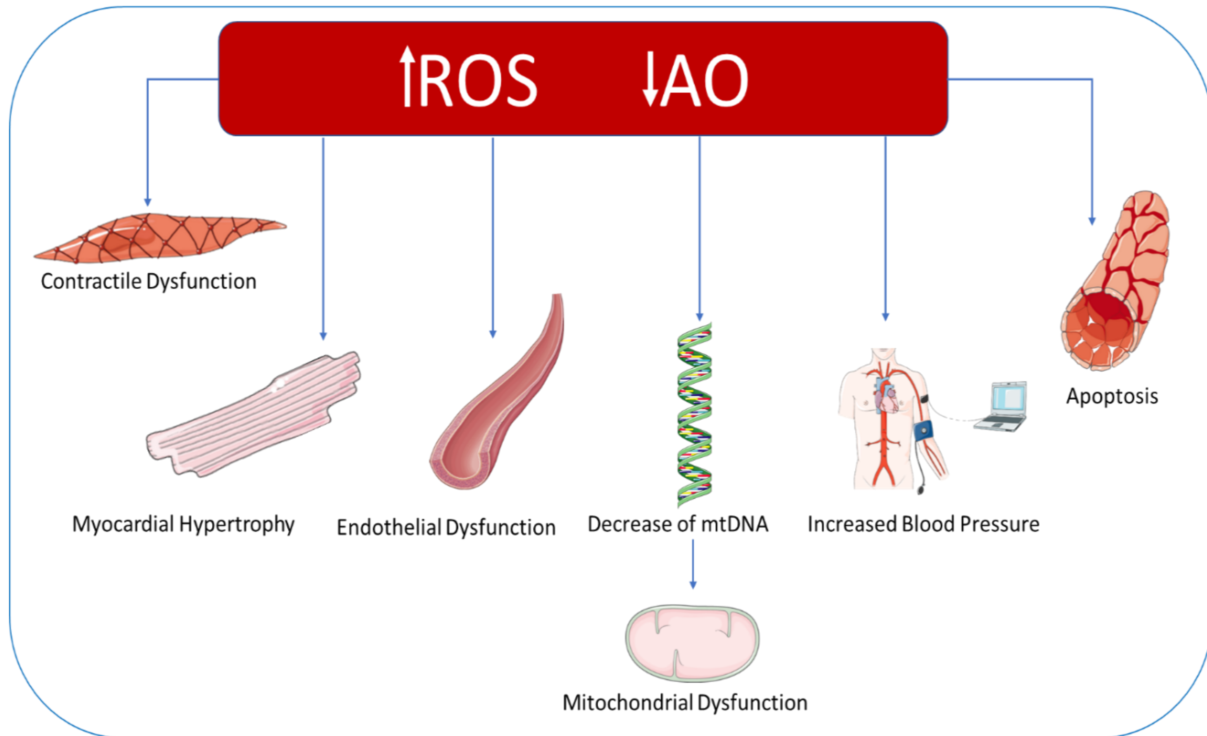

Figure 2: *Pathological roles of oxidative stress in cardiovascular tissues. ROS: reactive oxygen species, AO: antioxidants, mtDNA: mitochondrial DNA.*

1.2 Nitric oxide, the hero of the cardiovascular system

•NO is an endogenous gaseous radical synthesized in endothelial cells by the endothelial nitric oxide synthase (eNOS) from L-arginine, molecular O_2, NADPH, and other cofactors. The nitric oxide synthase (NOS) converts the amino acid L-arginine to produce L-citrulline and •NO. The metabolic fate of •NO involves its spontaneous oxidation into nitrite ions and nitrate ions in aqueous media, which are eliminated in urine. Once •NO is released from the endothelium, it diffuses in the bloodstream and into the vascular wall. In the vascular wall, it penetrates into vascular smooth muscle cells where it binds to the heme moiety of the soluble guanylate cyclase (sGC), which then dephosphorylates guanosine 5'-triphosphate (GTP) to form cyclic guanosine 3′,5′-monophosphate (cGMP).

cGMP in turn activates the protein kinase G (PKG), which leads to the phosphorylation of proteins responsible for the regulation of calcium concentrations, in turn, activating myosin phosphatase and reducing intracellular calcium levels, resulting in smooth muscle relaxation. The cGMP signalling triggered by •NO is terminated by phosphodiesterases (PDE) such as PDE5 that specifically degrade cGMP to GTP [85]. Therefore, through the •NO /sGC/cGMP pathway, •NO regulates the vascular tone

with vasodilatory properties. Moreover, •NO is also able to regulate platelet aggregation with anti-thrombotic activity, leukocyte adhesion for anti-inflammatory properties, and smooth muscle cell proliferation with anti-proliferative properties [86]. So, the continuous production and release of •NO from the endothelium plays an important role in vascular and cardiac homeostasis.
Despite its short half-life of a few seconds , •NO shows sustained bioactivity in the vasculature due to a storage and transport form of •NO in form of *S*-nitrosothiols (RSNO), such as *S*-nitrosohemoglobin (SNOHb), *S*-nitrosoalbumine (Alb-NO) and *S*-nitrosoglutathione (GSNO) (Figure 3) [84,87].

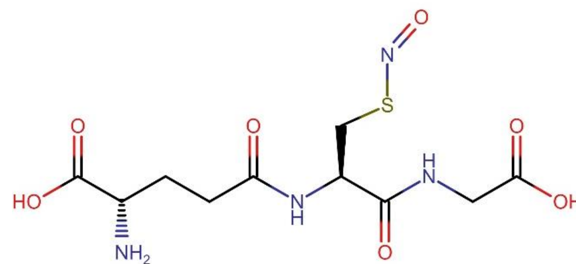

Figure 3*: The chemical structure of S-nitrosoglutathione (GSNO).*

S-nitrosation is the reversible addition of a •NO group to cysteine residues (Cys) in peptides or proteins, forming RSNOs. Protein *S*-nitrosation has been established as an important pathway by which •NO transmits its global cellular influence [88]. Furthermore, other types of modifications mediated by •NO have been studied, such as metal nitrosylation or tyrosine nitration, which is an irreversible addition of $ONOO^-$ on a tyrosine residue of a protein [89–91]. •NO can be released from RSNO by enzymes such as gamma-glutamyltransferase (GGT) specific for GSNO or redoxins like protein disulfide isomerase (PDI) or the thioredoxin (Trx) system to achieve its physiological activities (Figure 4) [92,93]. Moreover, in aortic smooth muscle cells, protein *S*-nitrosation has been widely evaluated to better comprehend its impact on vascular function. Further, protein *S*-nitrosation in human aortic smooth muscle cells (HuAoSMCs) can modify their contractile properties. For example, *S*-nitrosation of actin or the myosin light chain kinase leads to the relaxation of vascular smooth muscle cells and therefore vasodilation [94]. In addition, protein *S*-nitrosation can regulate the cellular redox balance and inflammation signalling pathways in HuAoSMCs, which moderate the activity of inflammatory mediators, leading to decreased inflammation and reduction of the progression of vascular diseases [92,95,96]. Moreover, our laboratory has demonstrated that GSNO presents antioxidant properties in vascular smooth muscle cells through *S*-nitrosation/transnitrosation processes [94]. Transnitrosation is the transfer of •NO to a protein protecting the affected Cys residue(s) of those proteins from oxidation.

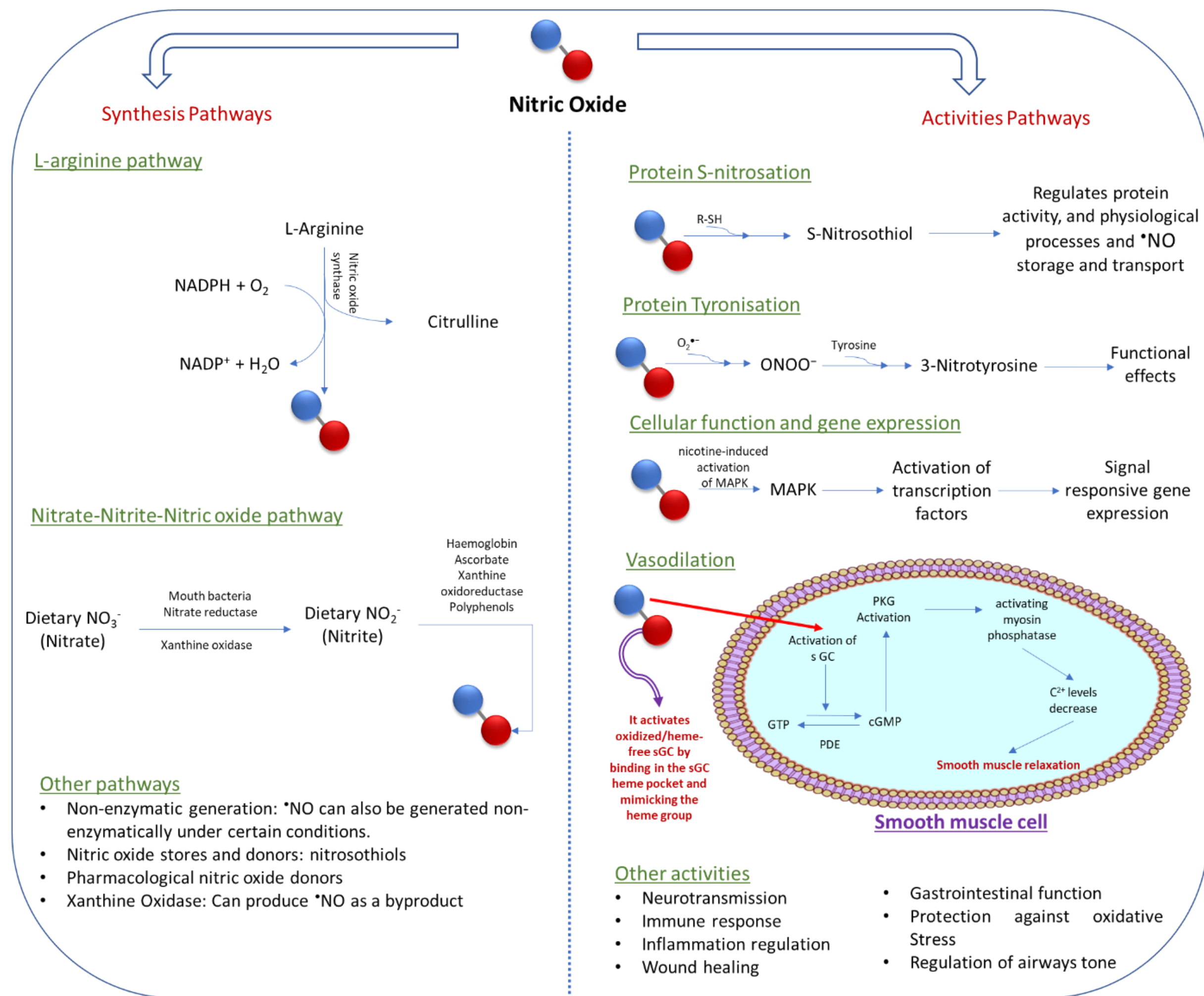

Figure 4: *The synthesis and effect pathways of •NO: nitric oxide, sGC: soluble Guanylate cyclase, GTP: Guanosine 5'-Triphosphate, cGMP: Cyclic guanosine 3',5'-monophosphate, PKG: protein kinase G, PDE: phosphodiesterases, $ONOO^-$: peroxynitrite, MAPK: mitogen-activated protein kinase, R-SH: Thiol, H_2O: water, O_2: dioxygen; $O_2^{•-}$: superoxide anion, NADPH: Nicotinamide adenine dinucleotide phosphate hydrogen, $NADP^+$: Nicotinamide adenine dinucleotide phosphate.*

1.3 *Protein S-nitrosation and the intriguing world of protein modification*

Indeed, the important role of •NO in the reversible modification of proteins through the *S*-nitrosation process might affect protein activity, protein-protein interactions, and protein location, influencing various redox-based signalling processes, including vasodilation [97]. *S*-nitrosation is a post-translational modification wherein a thiol is transformed into a nitrosothiol. While the terms nitrosation and nitrosylation are commonly used reciprocally, it is more accurate to reserve "nitrosation" for describing the formation of a nitroso group (R-NO). This process involves a one-electron oxidation from the •NO

radical. The term "nitrosylation," originally designed to draw an analogy to phosphorylation (the transfer of the phosphoryl group), is chemically misleading. Formally, the nitrosyl group is a •NO radical, and during nitrosation reactions, it is the nitroso group (not the nitrosyl group) that is transferred. Nitrosylation, on the other hand, specifically refers to the coordination of the nitrosyl group with a transition metal. Therefore, we prefer to use the term *S*-nitrosation to accurately describe the conversion of a thiol to a nitrosothiol [98]. Protein *S*-nitrosation relies on the balance between nitrosation and denitrosation. This dynamic process is crucial to prevent pathological events linked to nitrosative stress that can result from over-expression and generation of •NO and •NO-derived reactive species. $ONOO^-$ ions can react with lipids, DNA, and proteins *via* direct or indirect oxidative reactions. These reactions trigger cell responses that range from cell signalling modifications to oxidative/nitrosative injury. *In vivo*, $ONOO^-$ formation is implicated in cardiac pathogenic episodes [99]. Denitrosylases are enzymes involved in regulating *S*-nitrosation, denitrosylases may play a part in reducing nitrosative stress and to control signal transduction. Denitrosylases also contributes in setting levels of cellular nitrosylation, and phosphorylation. Currently only, two enzymatic denitrosating systems have been demonstrated to function in physiological contexts: the Trx/TrxR system and the glutathione (GSH)/GSNO reductase (GSNOR) system where only the Trx/Trx reductase (TrxR) system is able to release •NO whereas GSNOR reduces GSNO and metabolizes •NO into hydroxylamine (NH_2OH), (Figure 5) [95,96,100,101]. GSNOR, also known as aldehyde dehydrogenase 5, catalyzes the reduction of GSNO and generates the temporal, unstable molecule N-hydroxysulfinamide (GSNHOH) in the presence of nicotinamide adenine dinucleotide hydrogen (NADH). GSNHOH intermediate metabolite, which in the presence of glutathione (GSH) generates ammonia (NH_3) or hydroxylamine and forms glutathione disulfide (GSSG). The NADPH-dependent activity of glutathione reductase (GR) reduces GSSG back to GSH, which becomes again available for transnitrosation reactions. In an oxidative stress environment where GSH is very low GSNHOH forms glutathione sulfinamide ($GSONH_2$) via spontaneous rearrangement then generates glutathione sulfonic acid (GSSOH) and ammonium (NH_4) [102].

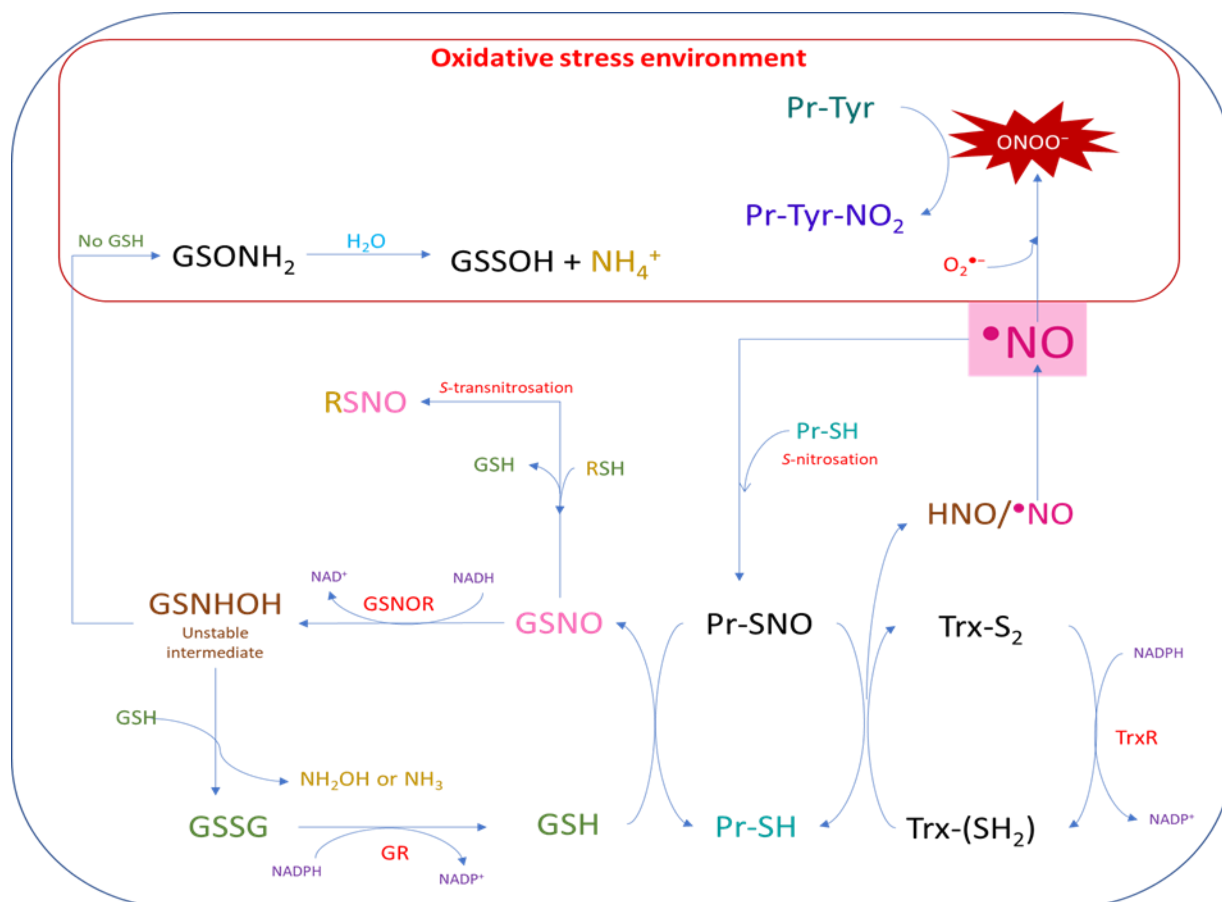

Figure 5: *S-nitrosation/denitrosation systems. Pr-SNO: S-nitrosated proteins, •NO: Nitric oxide, GSH: Glutathione GSNO: S-nitrosoglutathione, GSNOR: GSNO reductase, NADH: Nicotinamide adenine dinucleotide hydrogen NAD⁺: Nicotinamide adenine dinucleotide hydrogen, NADPH: Nicotinamide adenine dinucleotide phosphate hydrogen, NADP⁺: Nicotinamide adenine dinucleotide phosphate GSNHOH: S-hydroxysulfenamide, NH_3: Ammonia, NH_2OH: Hydroxylamine, GSSG: Glutathione disulfide, GR: Glutathione reductase, $GSONH_2$: Glutathione sulfinamide, GSSOH: Glutathione sulfinic acid, NH_4: Ammonium, TRX−$(SH)_2$: Reduced thioredoxine, HNO: Nitroxyl , TrxR: Trx reductase, Trx-S_2: Disulfide form of thioredoxine, $ONOO^-$: Peroxynitrite.*

Chronic or massive oxidative stress can exhaust GSH, which further accelerates the depletion of GSH and thereby the aggravation of oxidative stress. It is not definite, but GSNOR appears to preferentially utilize NADH for reducing GSNO, whereas GR utilizes NADPH more frequently. Furthermore, TrxR enzymes are important selenoproteins that, together with Trx and additional Trx-dependent enzymes, carry out several antioxidant and redox regulatory roles in cells. There are two main subtypes of Trx in mammalian cells: Trx1 mainly localizes in the cytoplasm a and Trx2 distributes in the mitochondria. Trx1 and Trx2 share an active motif, Trp-Cys-Gly-Pro-Cys, with major redox properties mediated by two

cysteines. Similar to GSH-induced transnitrosation forming GSNO, Trx facilitates transnitrosation, leading to SNO-Trx and concurrent reduction of S-nitrosated proteins.

S-nitrosated Trx eventually forms intramolecular SS bonds, releasing free •NO or nitroxyl (HNO) based on the degree of *S*-nitrosation in the TRX molecule [103].

Released •NO can be reused for intracellular •NO signaling. Intriguingly, Trx also releases HNO, whose functional importance is not fully understood, but studies suggest it activates biological functions similarly to •NO, acting as a secondary messenger. HNO shows beneficial effects in cardiovascular physiology, including platelet aggregation inhibition and vasorelaxation [104].

Therefore, GPx-like organoselenium molecules seem to be of great interest for their antioxidant and denitrosating activity releasing •NO. As already mentioned, GSNO presents antioxidant properties through the transnitrosation process. However, GSNO is the *S*-nitrosated form of the most abundant cellular thiol, GSH (Figure 6).

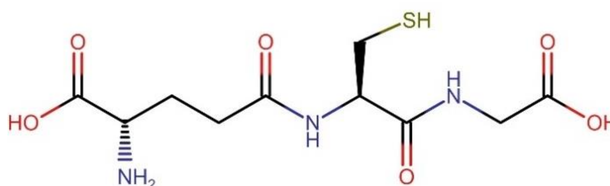

Figure 6: *Chemical structure of Glutathione (GSH)*

GSNO is the biological store of •NO in cells and a key player of •NO-dependent signal transduction. In addition, there has been an increased interest in GSNO as a potential therapeutic agent. GSH is a key antioxidant tripeptide (L-γ-glutamyl-L-cysteinyl-glycine) found in great concentrations (0.5 to 10 mmol/L) in every cell type. It has an important role in maintaining the cellular redox balance and in protecting against oxidative stress [105]. On the one hand, as already mentioned, it plays a role in the regulation of protein *S*-nitrosation. GSH can react with RSNOs to release •NO and GSSG, which regulates the levels and functions of RSNOs and can modulate the signalling and functional properties of RSNO-modified proteins [106]. This reaction might be catalysed by glutathione-*S*-transferases. On the other hand, as a cofactor of two different enzymes, GSH is involved in the detoxification of xenobiotics by glutathione-S-transferase through the *S*-glutathionylation process and in the reduction of peroxides by GPx enzymes. In addition, it has been recently discussed that the *Se*-nitrosation in selenoproteins such as GPx and TrxR to produce *Se*-nitrososelenocysteine (Sec−SeNOs) can be formed by direct transnitrosation from an RSNOs to a selenol, and has been proposed to play essential roles in signalling processes mediated by reactive nitrogen species and nitrosative-stress responses [107–109].

1.4 The guardians of the cell redox balance: The GPx Family

Maintaining the balance of cellular redox functions is essential in determining the cell's fate. In mammalian cells, GPx represent the most abundant family of proteins with a versatile role that affects cellular processes. A group of eight members of the GPx family named GPx1 to GPx8 are currently found in mammals. They are antioxidant enzymes with an important function in fighting oxidative stress and maintaining redox balance, protecting biomolecules such as proteins, amino acids, lipids, and DNA from oxidative damage. However, each member of the GPx family has its specificity of substrate and mechanism/site of action to maintain an adequate redox balance. GPx1-4 and GPx6 use selenocysteine (Secys) as the active centre to catalyse the reduction of H_2O_2 or organic hydroperoxides to water or corresponding alcohols. They employ GSH as a cofactor, which is oxidized to GSSG. The latter, in turn, is recycled to GSH by GR utilizing NADPH (Figure 7).

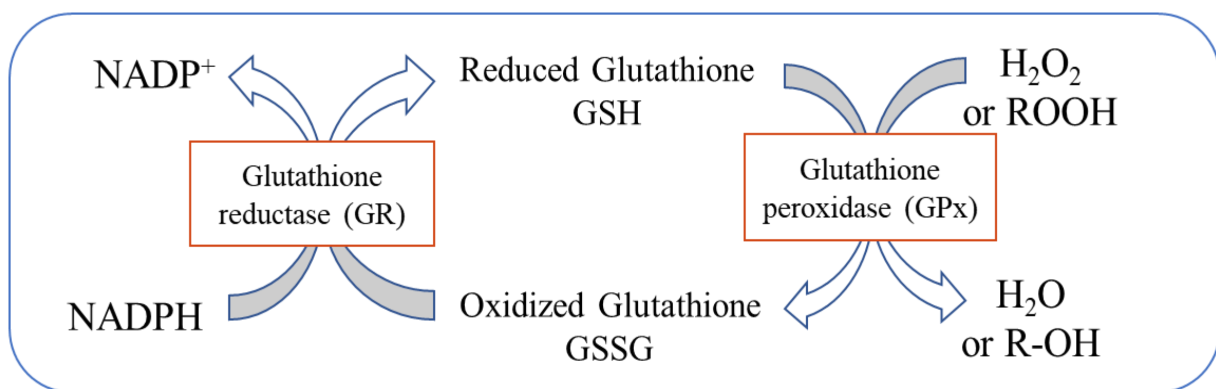

Figure 7: *GPx catalytic cycle in reducing peroxides. H_2O_2: Hydrogen peroxide, H_2O: Water, ROOH: Hydroperoxides, R-OH: Alcohol, NADPH: Nicotinamide adenine dinucleotide phosphate hydrogen, $NADP^+$: Nicotinamide adenine dinucleotide phosphate*

GPxs have also been shown to protect against oxidation mediated by $ONOO^-$, reducing its toxicity and maintaining the redox balance. In addition, GPx4 is the only enzyme in the GPx family that directly reduces lipid hydroperoxides. Interestingly, the active sites of GPx5, GPx7 and GPx8 do not contain Secys, but a Cys residue. GPx5 is mainly expressed in epididymal tissue and plays a role in protecting sperm from oxidative stress, whereas both, GPx7 and GPx8, are located in the endoplasmic reticulum and are important enzymes in the oxidative folding of endoplasmic reticulum proteins. With extensive research and a deep understanding of the role of the GPx family members in biology, redox balance has become the main interest of the GPx family [110,111].

1.5 Selenium is a key player in health and diseases

Article I: **Selenium Donors at the Junction of Inflammatory Diseases**

Rama Alhasan, Ammar Kharma, Pierre Leroy, Claus Jacob and Caroline Gaucher

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Selenium is an essential non-metallic trace element, and inadequate intake has been linked to various diseases, including serious conditions such as respiratory distress syndrome, myocardial infarction, renal failure (selenium overload) and chronic inflammatory diseases such as inflammatory bowel disease, rheumatoid arthritis and atherosclerosis (selenium deficiency). The primary source of selenium is the diet, derived from animal and cereal sources, and its intestinal absorption is limited to selenocysteine and selenomethionine and their incorporation into selenoproteins. This review discusses the relationship between selenium and inflammatory diseases and explores the potential of selenium nanoparticles and organoselenium molecules to compensate for dietary selenium deficiency. In the case of high selenium load, nanoparticles allow low-dose restoration of selenium bioavailability, while organoselenium molecules play a role in modulating their antioxidant or anti-inflammatory activities.

In fact, selenium plays a crucial role in the synthesis of selenocysteine, a central component of the catalytic activity of enzymes such as GPx. Selenium also plays several important roles in the body, including anti-mutagenic, anti-carcinogenic, antiviral, antibacterial and antifungal effects. Selenium is an important element with antioxidant and redox-modulating activities that can also stimulate the synthesis or release of •NO from endogenous *S*-nitrosothiols. Organoselenium molecules have the ability to enhance protein *S*-nitrosation and •NO mobilisation in aortic cells, which is of particular interest in CVDs. They promote vasodilation, improve endothelial function and have the potential to restore vascular homeostasis and prevent or treat various cardiovascular pathologies. These molecules, when combined with •NO donors, can protect cysteine (Cys) residues and together may offer effective therapeutic potential for CVD and other health problems [112–116].

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ARTICLE TYPE

Selenium donors at the junction of inflammatory diseases

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Abstract:

Selenium is an essential non-metal trace element, which bioavailability imbalance is associated with many diseases ranking from acute respiratory distress syndrome, myocardial infarction and renal failure (Se overloading) to diseases associated with chronic inflammation like inflammatory bowel diseases, rheumatoid arthritis, atherosclerosis (Se unload). The only source of selenium is the diet (animal and cereal sources) and its intestinal absorption is limiting for selenocysteine and selenomethionine synthesis and incorporation in selenoproteins. In this review, after establishing the link between selenium and inflammatory diseases, we envisaged the potential of selenium nanoparticles and organic selenocompounds to compensate a deficit of selenium intake from the diet. With high selenium loading, nanoparticles offer a low dosage to restore selenium bioavailability whereas organic selenocompounds can play on the modulation of their antioxidant or anti-inflammatory activity.

Keywords: Selenium, redox, inflammation, organoselenium, selenium nanoparticles, selenoproteins.

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1. INTRODUCTION

The selenium element (Se) is a member of the chalcogen group. It possesses six valence electrons responsible for its high avidity to attract two supplementary electrons. This is the case when selenium, like sulfur, reacts with metals and non-metals to form ionic compounds containing the selenide ion (Se^{2-}) like in FeSe and in Na_2Se. As a redox compound, selenium exists, in elemental and combined forms, under different oxidation states ranging from -2 (sodium selenide, Na_2Se,), 0 (elemental selenium, Se), +4 (sodium selenite, Na_2SeO_3,), to +6 (sodium selenate, Na_2SeO_4,). Selenium can also form two covalent bounds as in selenoproteins where it substitutes the sulfur element in cysteine or methionine to form selenocysteine and selenomethionine residues. In these amino acids, the selenol group (-SeH) confers a stronger acid capacity than the thiol group. Indeed, the pKa of the selenol group of selenocysteine is 5.5 compared to 8.3 for the sulfhydryl group of cysteine. So, at physiological pH, selenol groups are ionized whereas thiol groups are mostly protonated, modulating their catalytic activity [1].

The analogy between selenol and thiol groups extends to their interaction with nitric oxide (NO) or NO-derived species. Indeed, thiol groups are able to form a covalent bond with NO, through *S*-nitrosation process, producing *S*-nitrosothiols, a form of storage, transport and modulation of NO signaling pathways [2]. The selenol group of selenocysteine can also be *S*-nitrosated forming *Se*-nitrososelenol, inactivating enzymes like glutathione peroxidase (GPx) [3-5]. However, as the selenium-nitrogen bond energy (Se-NO) is weaker than the sulfur-nitrogen bond energy (S-NO), *Se*-nitrososelenols are more labile than *S*-nitrosothiols, and the *Se*-nitrososelenol species were not identified in biological samples. The hypothesis of the *Se*-nitrososelenols existence was confirmed by its *in vitro* synthesis using direct transnitrosation from a *S*-nitrosothiol, the *S*-nitrosoglutathione, to a selenol group bearing a Bpq group (5′,5′′′-bis(2,6-diisopropylphenyl)-2,6,2′′′′,6′′′′-tetraisopropylm-quinquephenyl-2′′-yl) enabling the stabilization of the *Se*-nitrososelenol formed. The weaker stability of *Se*-nitrososelenols compared to *S*-nitrosothiols was also exploited as a selenium driven catalysis of NO release from *S*-nitrosothiols for antithrombotic or antimicrobial properties [6-9].

The selenol group incorporated in selenoproteins like GPx and thioredoxin reductases (TrxR) participates in redox reactions. It shares several chemical properties with the thiol group with a higher reactivity than its sulfur analogue. Due to its biological significant role, the substitution of S by Se has been exploited for a long time in organic synthesis to propose organoselenium compounds mimicking GPx activity and regulating free radicals production in the organism [10-12]. In the last 4 decades, the re-discovery of Ebselen® (2-phenyl-1,2-benzisoselenazol-3(2H)-one), the leader of organoselenium compounds, for its antioxidant and therapeutic properties has renewed interest in the field. Indeed, Ebselen® is actually under investigation in clinical trial for the treatment of Meniere's disease (phase 2) in the US, and for bipolar disorder (phase 2) in the UK.

During the last few decades, an immense attention was directed towards selenium, due to its noticeable importance in human health for thyroid hormone synthesis, antioxidant defense and inflammation resolution [13-17]. As an essential non-metal trace element, selenium is incorporated through the diet under two main dietary forms such as selenocysteine from animal-sources and selenomethionine derived from animal-sources, and cereal products grown in Se-rich soil in some areas like in the US. Selenium intake in Europe is generally low, probably due to low levels in soil, in particular in the East of Europe. Furthermore, the selenium intake in the UK has decreased since 1970 [18]. The Food and Nutrition Board at the Institute of Medicine National Academies (US) set the recommended average intake of selenium to 45 µg /day for males and females between 19-50 years old [17, 19]. Higher intake levels around 1600 µg of Se/day have been associated with hair and fingernails loss, garlic breath. While a toxic intake, around 5000 µg of Se/day, causes acute respiratory distress syndrome, myocardial infarction and renal failure [17, 20]. An intake below 30 µg/day is a causative factor of cardiomyopathies such as Keshan disease, and is also believed to contribute to osteochondropathies like Kashin-Beck disease.

2. NATURAL OCCURRENCE AND ROLE IN HUMAN PHYSIOLOGY

Naturally occurring selenium exists under either inorganic or organic forms (Fig. **1**). The selenoprotein family represented the main organic form where the selenium atom is covalently linked to the carbon atom of the skeleton of the methionine or cysteine amino acids. The chemical role of selenium is based on the selenol group (-SeH) participating in redox reactions. The selenoprotein family included enzymes catalyzing these redox reactions like the so-called GPx catalyzing the reduction of hydroperoxides (ROOH) using reduced glutathione (GSH) as a substrate and the TrxR reducing thioredoxin (Trx) and taking place in the Trx system. Another organoselenium compound, the selenoneine (Fig. **1**), is the major form of organic selenium in tuna [21]. The blood of pacific mackerel and tilapia, as well as porcine kidney, the heart, gizzard and liver of chicken, and squid hepatopancreas also contained selenoneine and selenoproteins, whereas porcine liver contained only selenoproteins [21].

The selenomethionine (Fig. **1**) is naturally occurring in plants, in *Clostridium* (thiolases, beta-D-hydroxybutyryl-coenzyme A, beta-galactosidase) and in animals such as tuna, salmon, mussels, pork and beef [22-25]. Therefore, the selenomethionine, found in the diet, is converted to selenocysteine by cystathionine beta-synthase and cystathionine gamma-lyase *via* a selenocystathionine intermediate [26]. Then, the selenocysteine is metabolized by the selenocysteine beta-lyase releasing hydrogen selenide (H_2Se) considered as the central point in the metabolic conversion of organic and inorganic selenocompounds [26]. Hydrogen selenide is a colorless gas with strong reducing and acid properties with a pKa of 3.73. The Se-methylselenocysteine found also in the diet is converted into methylselenol (CH_3SeH) by the cystathione gamma-lyase [27]. Then the methylselenol is demethylated to form hydrogen selenide [28]. The selenite ion (SeO_3^{2-}) can be reduced either by the thioredoxin system (thioredoxin/thioredoxin reductase) to form hydrogen selenide or by GSH to form selenodiglutathione [29]. The latter is a substrate of glutathione reductase to produce glutathioselenol reacting with GSH to yield hydrogen selenide [29].

The adequate level of selenium in human plasma is between 60 and 140 µg/L. The only sources are found in the diet under selenite, selenate, selenocysteine or selenomethionine form (Fig. **1**). In humans, Se is incorporated as selenocysteines within 25 selenoproteins synthesized following the decoding of a UGA codon. Among the 25 selenoproteins, only 13 have known functions: GPx, TrxR, iodothyronine deiodinases, selenoprotein R (SelR) and selenophosphate synthetase 2 (SPS2). These proteins exert functions including the redox homeostasis, the redox regulation of intracellular signaling, and thyroid hormone metabolism. At least 12 selenoproteins are oxidoreductases, with a selenocysteine catalytic redox-active residue [30, 31]. The oxidoreductases such as the Se-dependent glutathione peroxidases family take part in the reduction of H_2O_2 and hydroperoxides of lipids and phospholipids.

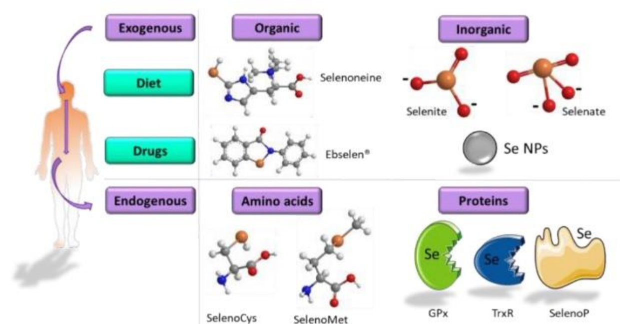

Fig. (1). The different exogenous sources of selenium found either in the diet or as drugs to form the human endogenous store and selenium-based catalytic activity.

3. INTESTINAL ABSORPTION AND TRANSPORT TO TISSUES

The absorption of selenium from diet intake takes place in the small intestine [32]. The selenocysteine and selenomethione are taken up into enterocytes by the selenoamino acids transporters B0 and $b^{0,+}$ rBAT [33]. The SLC13 cotransporters NaS1 (SLC13A1) and NaS2 (SLC13A4) have been proposed as selenate transporters [34]. The selenite transporter remains to be identified.
The selenoprotein P (SelenoP or Sepp1) is present in the jejunum and the colon part of the intestine and was shown to be secreted into the culture medium across the basolateral membrane in a model of intestinal barrier [35]. The secretion of SelenoP by enterocytes might be involved in the absorption and the transport of selenium from the intestinal epithelium to the liver *via* the portal circulation. Indeed, the SelenoP is a vital component for selenium transport, metabolism and downstream selenoproteins synthesis. The primary structure of SelenoP contains 10 selenocysteine residues. The smaller C-terminal domain includes nine selenocysteines providing the selenium transport capacity and the N-terminal domain includes one selenocysteine residue [36, 37]. The thioredoxin folding of the N-terminal domain handles a thiol-redox function [38, 39]. The SelenoP is produced in the liver, where selenium is also metabolized, and secreted into the plasma for selenium transport and distribution to distant tissues [37]. SelenoP transports up to 60% of the selenium in the plasma representing the major form of selenium transport [40]. The SelenoP levels are sensitive to the level of selenium in the diet [41-43]. Therefore, the conjunction between plasma selenium concentration, SelenoP levels and GPx activity usually evaluates the whole body selenium status.

Different receptors are able to mediate the uptake of SelenoP from the plasma to tissues, like testis, kidney, and the brain. Indeed, in the kidney, a member of the LDL receptor-related protein (LRP) family, the megalin (Lrp2) is believed to mediate SelenoP uptake from the glomerular filtrate [44]. Another lipoprotein receptor, the apolipoprotein E receptor-2 (ApoER2, LRP8) was found to be associated with SelenoP in the brain and in the testis [45]. The binding to the apoER2 induces the endocytosis of the apoER2-SelenoP complex, which will be then degraded in the lysosome to release selenocysteine allowing for the synthesis of new selenoproteins [46, 47].

In addition to its role in Se transport, the SelenoP possesses antioxidant activity by reducing peroxynitrite ions and lipid hydroperoxydes, and also by enabling low-density lipoproteins (LDL) oxidation *in vitro* [38, 48, 49]. The SelenoP also acts as a Se-donor protein that contributes to the protection of endothelial cell against oxidative stress [50]. Indeed, SelenoP is internalized by endothelial cells using the ApoER-2 and the megalin, and the released selenium is incorporated into GPx and TrxR increasing their antioxidant activities [44, 45].

To date and to the best of our knowledge, no specific storage form of selenium in tissues has been identified. However, the selenium blood levels of women moving from a high selenium intake country to a low selenium intake country, took over a year to reach the local level showing the ability of the human body to store selenium [51]. In analogy to the storage form of NO upon sulfur reactivity, the *S*-nitrosoproteins, the selenoproteins containing selenocysteine or selenomethionine residues can be envisaged as a storage form of selenium.

4. THE ANTI-INFLAMMATION POWER OF SELENOENZYMES

4.1. Glutathione Peroxidases

The GPxs are a family of eight enzymes providing antioxidant protection against hydroperoxides [52, 53]. Apart from GPx4, which is a monomer, GPxs are tetramers containing about four grams of selenium atoms per one mole [54]. The cytosolic GPx1 (EC 1.11.1.9) protects against hydrogen peroxide and lipid hydroperoxides, such as those from esterified phospholipids in cell membranes [55]. The extracellular GPx2 (EC 1.11.1.9) and GPx3 (EC 1.11.1.9), with similar tetrameric structures to GPx1, operate in different compartments, such as the gastrointestinal tract and the plasma, respectively [56]. GPx2 is exclusively present in the gut of humans and rodents where GPx1 and GPx4, as ubiquitous enzymes are also expressed [57-59].

GPx2 was found to be the major antiapoptotic GPx in the colon, and GPx1 can partially compensate for the loss of GPx2 activity [60]. The secreted glycoprotein GPx3 is the second most abundant selenoprotein in the plasma. The phospholipid hydroperoxide glutathione peroxidase, GPx4 (EC 1.11.1.12) represented by the cytosolic (cGPx4), nuclear (nGPx4) and mitochondrial (mGPx4) isoforms has a high specificity for a wide range of lipid hydroperoxides linked to the plasma membrane such as phospholipids and cholesterol. The oxidation of arachidonic acid by lipoxygenases produces

pro-inflammatory intermediates, the 5-hydroperoxyeicosatetraenoic acids (HpETEs). All these HpETEs can be reduced by GPx4 to generate HETE [61-63]. Furthermore, GPx4 inhibits the NF-κB pathway limiting cyclooxygenase and lipooxygenase expression [64]. All these findings indicate the GPx4 activation as beneficial for inflammation resolution [65-67].
More recently, GPx4 has been implicated in ferroptosis [68, 69] and its activation has been shown to promote inflammatory resolution [67, 70, 71].
GPx5, GPx7 and GPx8 are not selenoproteins [72, 73]. ~~The~~ GPx 6 is mainly expressed in olfactive epithelium and embryonic tissues [74]

4.2. Thioredoxin Reductases

The Trx system composed by two enzyme families, the Trx and TrxR, is NADPH dependent as it is driven by NADPH-dependent oxidoreductases namely, the cytosolic TrxR1 (EC 1.8.1.9) and the mitochondrial TrxR2 (EC 1.8.1.9). The system is also selenium-dependent as these enzymes include a catalytic selenocysteine residue making them members of the selenoprotein group [75]. The cytosolic TrxR1 enzyme can also be found in the nucleus with an alternative splicing, where it can modulate the activity of transcription factors, such as the estrogen receptors [76]. Another alternative splicing form is targeted to cell membranes, where it co-localizes with membrane rafts and has the capacity to promote the formation of filopodia for cell movements [77, 78]. Mammalian TrxRs display very broad substrate specificities and reduce many low molecular weight compounds [75]. The primary substrates of TrxR1 and TrxR2 are Trx1 and Trx2, respectively, and most actions of the mammalian thioredoxin system are likely to be carried out by the activities of Trx1 and Trx2 [75, 79].
Several studies indicate that the Trx system is likely to modulate the balance between protein *S*-nitrosation and denitrosation reactions, and thereby to control the NO signaling pathways [80-83]. However, the exact mechanism by which the Trx system regulates NO signaling is still unclear. Intriguingly, *S*-nitrosoglutathione (GSNO), which is synthesized by the spontaneous formation of a bond between NO and the thiol function of GSH is a potent substrate for TrxR, resulting in the release of GSH and NO [84]. Protein *S*-nitrosation, which is a reversible post-translational protein modification that involves the attachment of a NO moiety to a sulfhydryl group, can modulate the activity of proteins and is involved in redox based cellular signaling [85]. Indeed, under oxidative stress, GSNO increases the content of reduced thiols group on proteins as well as protein *S*-nitrosation highlighting, the protective role of the *S*-nitrosation process for thiol groups undergoing oxidation [86]. Furthermore, the inhibition of TrxR was shown to increase protein *S*-nitrosation and to impair acetylcholine-induced vasorelaxation in a model of angiotensin II (AngII)-induced hypertensive C57BL/6 mice [87]. All together, these data show the importance of the Trx system in *S*-nitrosation and denitrosation processes to render NO bioavailable for relaxation. Indeed, at the vessel level, protein *S*-nitrosation modulates the vasoreactivity of aorta through the formation of a releasable NO store [88]. Furthermore, an increase of the Trx expression and transcription was observed in endothelial cells and macrophages in human atherosclerotic plaques [89]. This induction was shown to be dependent on NO in endothelial cells.

5. INFLAMMATION AND SE DEPLETION-ASSOCIATED PATHOLOGIES

Chronic inflammatory disorders such as cystic fibrosis, inflammatory bowel diseases (IBDs), rheumatoid arthritis and cardiovascular diseases (CVDs) have been associated with a decrease in selenium status compared to healthy patients [90]-[91, 92]. The sensitivity of selenoproteins to selenium deficiency is unequal presupposing for an unequal supply of selenium to all tissues [93]. Indeed, the expression of TrxR1 and GPx4 (referred to as housekeeping selenoproteins) are less sensitive to selenium deficient diet than the expression of GPx1 and selenoprotein R, known as methionine sulfoxide reductase B1 (referred to as stress-related selenoproteins).

5.1. Intestinal Bowel Diseases

The IBDs such as Crohn's disease and ulcerative colitis are disabling pathologies affecting young patients. They undergo high recurrence of acute inflammatory episodes all over their life, and this may finally lead to colorectal cancer. Most of the current therapeutics have a low efficiency/cost ratio, and act by down regulating chronic inflammation in the intestinal mucosa but cannot cure the disease. Since the 1980s, significant reductions of selenium levels in serum have been identified in both child and adult ulcerative colitis and Crohn's disease patients under quiescent disease or during crisis [94-98]. Furthermore, serum levels of selenium and SelenoP inversely correlate with crisis duration, ulcerative colitis severity, and Crohn's disease activity index [97, 99]. During the acute and recovery phase of colitis in dextran sulfate sodium (DSS)-treated mice, the anti-inflammatory cytokine Il-22 through STAT3 transcription factor upregulates the GPx2 [100]. Furthermore, selenium-deficient diet amplifies colitis and decreases the GPx plasma activity [101]. These findings indicate that GPx2 might be important for the resolution of inflammation. As these results were obtained with a DSS-induced colitis in mice having a standard diet, the decrease in selenium levels was attributed to altered dietary intake or nutrient absorption during colitis. Therefore, selenium is a biomarker for IBDs activity index and severity. Selenium supplementation by sodium selenite was shown to attenuate COX as well as myeloperoxidase mediated inflammation in both DSS-induced and 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis models, respectively [102, 103]. However, the kinetic as well as the selenium supplementation form seem to be critical points.

Indeed, long-term sodium selenite supplementation during colitis crisis had no effect on colitis development whereas short-term selenite supplementation resulted in a more severe colitis [104]. Furthermore, selenium nanoparticles administrated during DSS-induced colitis were shown to be protective especially on body weight loss, and colonic inflammatory damage [105].

5.2. Rheumatoid Arthritis

Rheumatoid arthritis, a chronic and autoimmune inflammatory disease, affects 1% of the population and is associated with significant morbidity and increased mortality [106]. The inflammation of tissue, mainly joints, with inflammatory cells infiltration and pro-inflammatory cytokine secretion are the main characteristics of this disease. The main treatments of rheumatoid arthritis are monoclonal antibodies directed against cytokines (Il-6, TNF-α) or antagonists of Il-1 and TNF-α receptors. However, these treatments are of high cost, administered by parenteral route and with a high inter-patient variability of efficacy. Several recent meta-analyses associate a low level of selenium in serum with autoimmune diseases such as rheumatoid arthritis [107, 108]. This low level of selenium may be a risk factor for inflammation and initiation of autoimmunity. Indeed, a 6 to 12 weeks duration of selenium deficient diet was shown to exacerbate the arthritic manifestations in a rat model of adjuvant-induced arthritis [109]. In this model, the supplementation with an inactive yeast (*Saccharomyces cerevisiae*) enriched with organic selenium resulted in a decrease in oedema and hyper-nociception [110]. The anti-inflammatory power of the enriched yeast extract was also confirmed in an antigen-induced arthritis model with a less recruitment of inflammatory cells in knee joints and a decrease of pro-inflammatory cytokines secretion in the peri-articular tissue [110]. Moreover, a sufficient intake of selenium from the diet limits the complications and improves the survival of patients [108, 111]. This phenomenon was assigned to the anti-inflammatory properties of selenium. Thereby, patients receiving anti-rheumatic treatment (methotrexate monotherapy or anti-TNF therapy with or without methotrexate) showed an increase in serum selenium concentration, still below the level that might be necessary for optimal CVD protection, after only six weeks of treatment [112].

5.3. Atherosclerosis

Atherosclerosis, an inflammatory disease associated with oxidative stress, develops at the bifurcation of large arteries through the infiltration of LDL in the vascular wall. To date, life-style improvement is the primary prevention of atherosclerosis. However, for patients with high CVD risk, statins, lowering the concentration of circulating LDL-cholesterol, are used to stabilize atherosclerotic plaques. Nevertheless, morbidity and mortality from atherosclerotic vascular disease remain substantial. Some clinical trials assessed the role of selenium supplementation as a prevention of CVD regarding its antioxidant effect. Some of these trials cannot support this preventive property whereas CVD risks were inversely correlated with selenium status, including SelenoP concentration [91, 92]. Nevertheless, these trials dealt with a selenium supplementation dose, alone or in association with other antioxidants, increasing the selenium status higher than the physiological concentration (60–140 µg/L). It was the case for the SU.VI.MAX (SUpplémentation en VItamines et Minéraux Anti-oXydants), which failed to show a real benefit of a cocktail of antioxidant supplements (including 100 µg/day of selenium) to prevent neither CVDs nor cancer [113]. The SU.VI.MAX 2 Follow-up Study (2007-2009) assessing healthy aging from the first SU.VI.MAX trial showed a benefit of the supplementation in the prevention of CVDs for old persons with a low baseline selenium status [114]. Indeed, a low dose of selenium decreased the total and non-HDL cholesterol concentrations, whereas the effect was non-significant for a high-dose supplementation (300 µg/day) [115]. Lipids, cytokines and antioxidants modulate GPx4 expression and activity in endothelial cells that in the presence of adequate selenium concentration, may favour the protection against pro-atherogenic processes [116]. Indeed, the transgenic over-expression of GPx4 significantly reduces lipid peroxidation in atherosclerosis and ischemia–reperfusion mouse models [117,118]. Furthermore, the intestinal GPx was shown to prevent the intestinal absorption of oxidized lipids [119] which was confirmed in a preclinical study where hamsters fed with hypercholesterolaemic diet and supplemented with selenite lowered their concentration of cholesterol in plasma [120]. On the contrary, in the National Health and Nutrition Examination Survey III in the US, a higher risk of diabetes (57%) was observed for the highest quintile of serum selenium (147 µg/L) compared with the lowest quintile (105.9 µg/L) [121]. Therefore, a better understanding of the relationship between selenium concentration and CVDs requires a better understanding of the role of selenoproteins in the cardiovascular system and an adequate supplementation of selenium. However, the form as well as the selenium loading of the supplementation linked with its bioavailability is of great importance for a proper selenium supply.

6. SELENODONORS: CHEMICAL DESIGN TO ACHIEVE PHARMACEUTICAL LOAD SPECIFICATIONS

The therapeutics for chronic inflammatory disorders, which are associated with a depletion of selenium bioavailability, have low efficiency. Therefore, ~~the~~ selenium supplementation, by increasing antioxidant activity and limiting inflammation, is an alternative therapy, or treatment, for patients, alleviating some of such pathologies-associated symptoms.

6.1. Selenium Nanocarriers

Nanocarriers are significant in the clinical field due to their particular properties to improve drug bioavailability and imaging capabilities. Various inorganic and organic nanomaterial-based drugs have been approved by drug agencies like Food and Drug Administration [122]. They are indicated in the treatment of cancer (35%) because they present in that case a high benefit/risk ratio, and in inflammation, immune and pain diseases (18%) and next, infection (12%).

Selenium nanoparticles (SeNPs) have not yet reached the therapeutic marketed field despite their numerous related preclinical reports (recently extensively reviewed). They mainly present a spherical shape with a diameter ranging from 10 to 100 nanometers. Thus, they are bigger than selenoproteins and the mechanisms involved in their physiological barrier crossing, transport, and cell uptake are comparable to other kinds of NPs. They present low cytotoxicity when compared to other kinds of NPs. They are mainly synthesized *via* reduction of selenium oxide salts either by using a chemical reagent (*e.g.* borohydride natrium) or by taking advantage of the biochemical machinery of microorganisms. In this latter case, the resulting SeNPs are more difficult to characterize by classical analytical methods. Most of the reported SeNPs exhibit antibacterial properties as silver, gold, zinc and iron NPs do with hypothesized oxidative stress mechanisms. Remaining redox properties of selenium could be expected in SeNPs giving rise to their remarkable properties in a broad spectrum of applications: antimicrobial, wound healing, anticancer, antifungal, and antiparasitic applications. However, it appears that the molecules (antiviral cytotoxic chemotherapeutic drugs, antioxidants) decorating SeNPs are most of the time responsible of the observed properties [123-126]. Moreover, other molecules are linked to SeNPs to enhance their targeting effects such as acetylcholine, which binds to the corresponding receptor on bacterial cell membranes [126].

In conclusion, SeNPs demonstrate a wide range of applications but they still need more preclinical investigations to persuade of their real clinical efficiency, especially in the field of inflammation and related diseases like cardiovascular ones [127]. Recent reports have introduced innovative biomaterials including organoselenium compounds, such as Ebselen® exhibiting antithrombotic and antimicrobial properties. These biomaterials were grafted on vascular stents to help and regulate NO level [8, 9, 128]. For example, when immobilizing selenocystamine on vascular stents, a GPx-like activity appears permitting a catalytic release of NO from *S*-nitrosothiols, thus inhibiting platelet activation and aggregation, and exhibiting anti-proliferation properties[8].

6.2. Organic Selenocompounds

Organic selenocompounds were ignored for long time, with minimum focus found in literature until 1970. This slow development is directly linked to the unpleasant reputation of these compounds, such as toxicity, instability of certain derivatives as well as the general belief that the chemistry of selenium would be more or less similar to that of sulfur compounds. However, organic selenocompounds have grown in a noticeable manner in the last three decades, probably because of their also substantially greater bioavailability than that of inorganic selenium forms [129, 130]. Since the anti-inflammatory and anti-oxidant properties of selenium are of clinical importance, nutritional interventions with selenium donors to prevent autoimmune or inflammatory diseases complications are considered.

Selenium dioxide (SeO_2) for instance was predominantly employed in organic synthesis until the early 1970s. Moreover, this interest in organic selenocompounds rapidly grew and several useful reactions and processes were discovered, with many compounds and derivatives synthesized [131, 132]. Selenium is now privileged in plenty of organic synthesis as an electrophile, nucleophile or even as a radical in a chemo-, regio- and stereo-selective manner [133, 134]. Further, the importance of selenium chemistry arose from recent interest in semiconductor metal selenide nano-materials [135-137]. However, those compounds have a significant medical importance, due to a strong antioxidant activity, anti-inflammatory and analgesic effects, that is opioid-independent and relies on Ca^{2+}, transient receptor potential, and mitochondrial modulation [138].

The medical potency of organic selenocompounds has led to many developments of selenium-based pharmaceutical compounds that are potential anticancer, antioxidant, and antimicrobial agents. Historically the simplest organoselenium compounds synthesized were selenols, selenides and diselenides but they were unstable and difficult to purify. Therefore, scientists are concentrating on the improvement and the development of selenium derivatives [139-141]. Currently, families of organic selenocompounds, such as seleninate esters, selenenic anhydrides, and pyrimidyl based diselenides are developed for their antioxidant activity, while other organic naphthyl based selenocompounds proved antithyroid properties [142-144]. These strategies produced groups and families of compounds with various biological activities such as anti-oxidant/anti-inflammatory (GPx activity) and anti-microbial activities [145, 146] (Table **1**).

Table **1**: The chemical families of synthesized selenocompounds and their biological activities.

Biological activity	**Chemical structure the family is based on**	**References**
GPx mimicking	-Nicotinoyl based -Spirodiazaselenurane based	[147-149]
Anti-microbial	-Carboxylic acid based	[150]

	-Imidazo[1,2-*a*] pyridine based	[151]
Anti-inflammatory	-Salicylic acid based diselenides	[146]

The anti-inflammatory activity was also seen with 1,3-selenazol-4-one derivatives, which inhibit the expression of inducible NO synthase (iNOS) in LPS-induced BV-2 cells. Furthermore, selenazolidine molecules, prodrugs of selenocysteine, were used to deliver selenium to inhibit inflammation [152]. The p-chloro-phenylselenoesterol proved anti-inflammatory and anti-oxidant effects on TNBS-induced IBDs in mice. The seleno-imine library was used to synthesize the 2-((1-(2-(2-(2-(1-(2 hydroxybenzylideneamino) ethyl) phenyl) diselanyl) phenyl) ethylimino) methyl) phenol with atheroprotective effect [153].

From a chemical perspective, small organic selenocompounds may indeed be modelled on naturally occurring selenoneine, and they may also provide additional benefits, such as a higher aqueous solubility and GPx-activity than Ebselen® (Fig. **1**)

CONCLUSION

Selenium as an essential oligoelement has a pivotal role in physiology as well as physio-pathological situations linked to redox as well as inflammation control. Small variation of its concentration leads to huge consequences varying from pro-inflamatory situation in several diseases such as atherosclerosis, bowel diseases and rheumatoid arthritis for selenium depletion, to cancers for selenium overloading. This review focused on the pro-inflammatory diseases linked to selenium depletion and on how to restore selenium bioavailability upon organic and inorganic selenium supplementation. The scope of this task is huge, as selenium has to be bioavailable and keep its redox properties. The emerging field of SeNP might open new possibilities for selenium supplementation.

CONFLICT OF INTEREST

No conflict to declare

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1.6 Exploring reactive selenium species: Redox power unleashed

Organoselenium molecules are either electron donors or mimics of selenoenzyme catalytic activity. They have garnered considerable attention due to their potential therapeutic benefits related to cellular antioxidant defence mechanisms, in particular GSH and RSNOs. These organoselenium molecules are of special interest because of their antioxidant and cytoprotective properties, which could enhance the body's ability to counteract oxidative stress. One of the main pathways in which organoselenium molecules exert their intriguing influence is by modulating the levels of GSH and RSNOs within cells. This involves a direct effect on the biosynthesis of GSH, by stimulating or upregulating specific enzymes due to providing selenium to the body. Two key enzymes in this process are gamma-glutamylcysteine synthetase and glutathione synthetase [117–119].

The consequence of this enhanced GSH synthesis is the restoration of the delicate balance between reduced GSH and its oxidized form, GSSG. Maintaining this balance is crucial because it directly affects the overall cellular redox status. A higher GSH/GSSG ratio is indicative of a reduced (antioxidant) environment, which is vital for protecting cells from oxidative damage and maintaining their normal functions.

Organoselenium molecules can also prevent nitrosative stress *via* their antioxidant and radical scavenging properties. In addition, they can influence •NO production and availability and prevent its degradation in smooth muscle cells by enhancing the activity of endothelial nitric oxide synthase (eNOS). Adequate •NO levels can promote *S*-nitrosation of proteins involved in vasorelaxation, leading to the desired effects on vascular function [120–122]. Organoselenium molecules can also interact with proteins and affect their *S*-nitrosation. These molecules may aid the transfer of •NO to specific cysteine residues, leading to protein *S*-nitrosation [123,124]. By enhancing antioxidant defence mechanisms and modulating redox signalling pathways, organoselenium molecules may help counteract the harmful effects of oxidative stress in cardiovascular pathologies [125,126]. Indeed, (2-phenyl-1,2-benzisoselenazol-3(2H)-one) (Figure 8), also known as ebselen, and its derivatives have been used to coat a medical device releasing •NO from blood circulating RSNOs in the presence of cysteine [127].

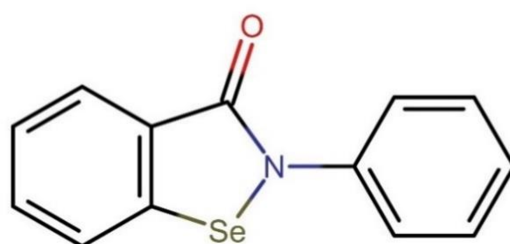

Figure 8: Chemical structure of Ebselen

Recently, there have been several efforts made in investigating and studying selenium-containing molecules that possess antioxidant and redox modulation activity, such as selenoneine, 2-selenyl-N_{α},N_{α},N_{α}-trimethyl-L-histidine, a selenium analogue of ergothioneine. It was determined as a potent antioxidant in tuna tissues and blood. This molecule has a unique chemical structure and is the first of a natural heterocyclic molecule derived from animals with a selenoketone motif.

Article II: **Selenoneine: A Unique Reactive Selenium Species from the Blood of Tuna with Implications for Human Diseases**

Rama Alhasan & Muhammad Jawad Nasim & Claus Jacob & Caroline Gaucher

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Aim of the study: The trace element selenium is found in many foods, from cereals to Brazil nuts. In humans, this chalcogen is essential for a number of physiological processes. A few years ago, selenonein, a rather unusual derivative of selenohistidine, was isolated from tuna.

Recent results: While there are a limited number of small natural selenium molecules, selenonein can be produced in large quantities in genetically modified microorganisms and by chemical synthesis. Due to the rare selenol/selenone tautomerism, this molecule exhibits unique redox properties and interesting biological activities, ranging from classical antioxidant activity to the interaction and subsequent protection of metal ions.

Conclusion: Selenone could be a promising initial basis for a new generation of selenium supplements and chemopreventive agents.

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Selenoneine: a unique Reactive Selenium Species from the blood of tuna with implications for human diseases

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Abstract

Purpose of review The trace element selenium is found in many dietary components, from grains to Brazil nuts. In humans, this chalcogen is essential for many physiological processes. A couple of years ago, selenoneine, a rather unusual seleno-histidine derivative, has been isolated from tuna.

Recent findings Whilst there is a limited number of other naturally occurring small selenium compounds, large quantities of selenoneine can be generated in genetically engineered microorganisms and *via* chemical synthesis. Due to a rare selenol/selenone tautomerism, this compound exhibits unique redox properties and promising biological activities, which range of traditional antioxidant action to the interaction and subsequent protection of metal ions.

Summary Selenoneine may indeed provide a promising lead for a new generation of selenium supplements and chemopreventive agents.

Keywords antioxidants, cardiovascular diseases, Reactive Selenium Species, selenol/selenone tautomerism, selenoneine, tuna

1. Introduction

Selenium is a chalcogen trace element essential for animal and human health (1, 2••). It is found in various plants, such as garlic, and a range of vegetables and legumes. Important sources of dietary selenium include beans, peas, nuts, seeds, meat and soy products (3-7). Grains and Brazil nuts, in particular, represent a prominent source of dietary selenium, and the exact levels of selenium present in these plants often depend on the content of selenium in the respective soils. In humans, the recommended dietary intake of selenium stands at 50 to 70 μg per day for an adult, yet real levels of intake vary significantly geographically, from just 7 μg per day in some regions to almost 5,000 μg per day in others (8). Unlike selenium, its "neighbour" in the Periodic Table, whose daily recommended uptake in terms of sulfur containing amino acids (*i.e.* cysteine and methionine) is 3 - 4 g per day which is fairly uncritical in the short term, an imbalanced dietary intake of selenium may swiftly result in serious health impediments. Selenium deficiency, for instance, is the main cause of Keshan and Kashin-Beck diseases (9, 2••). In contrast, an excess of selenium (selenium poisoning and selenosis) may also result in symptoms ranging from garlic breath, which is due to the excretion of hydrogen selenide (H_2Se) and volatile metabolites dimethyl selenide and trimethylselenonium *via* exhalation (10). Thus, selenium is often referred to as an "essential poison", a phrase reflecting rather nicely the delicate balance at the centre of an appropriate nutritional selenium uptake (11, 12, 2).

Indeed, regions particularly rich in selenium, where the element in its various chemical forms progresses from the soil into plants, animals and eventually humans, have seen the risks of a "selenium overload" (13). In the

Punjab region of India, for instance, daily intakes of 750 to 5,000 µg per day are not uncommon and have been associated with an increased risk of hair loss, discoloured or lost fingernails and staining of teeth (14, 15).

In contrast, selenium deficiency found in "selenium deficient regions", such as selenium-deprived regions of central Serbia, the belt stretching from north-east to south-central China and selenium nutrient-depleted sub-Saharan Africa, has resulted in attempts to supplement food and also soils with selenium in order to increase the daily selenium uptake of the population (16).

From a more chemical perspective, the controlled intake of selenium is also not entirely straightforward. At closer inspection, there are various selenium compounds present in foodstuffs. They range from the more common selenium containing amino acids selenocysteine (SeCys) and selenomethionine (SeMet) found, for instance, in nuts, grains and animals, to more "exotic" Reactive Selenium Species", such as methyl-*Se*-selenocysteine (cysSeMe) found in plants (*e.g.* garlic, astragalus and onions) and selenocystine (cysSeSecys) detected primarily in animals (Figure 1). Even inorganic selenite (SeO_3^{2-}) is on occasion considered as a decent source of nutritional selenium, despite the fact that this inorganic selenium species is highly reactive chemically and has been associated with damage to human DNA (17, 2).

Indeed, the "nutritional chemistry" of selenium is complicated, and each of the selenium compounds found in the diet exhibits its own reactivity and profile of biological activity. As part of this review, we will therefore consider the redox behaviour of some of the most prominent selenium compounds found in our diet. We will introduce and subsequently focus on the rather unusual compound selenoneine (2-

selenyl-N_{α},N_{α},N_{α}-trimethyl-l-histidine). This selenium-containing histidine derivative has been isolated for the first time about a decade ago from tuna and since has received a certain prominence as a possible nutritional supplement, placing fish firmly on the table of selenium and potential benefits for human health.

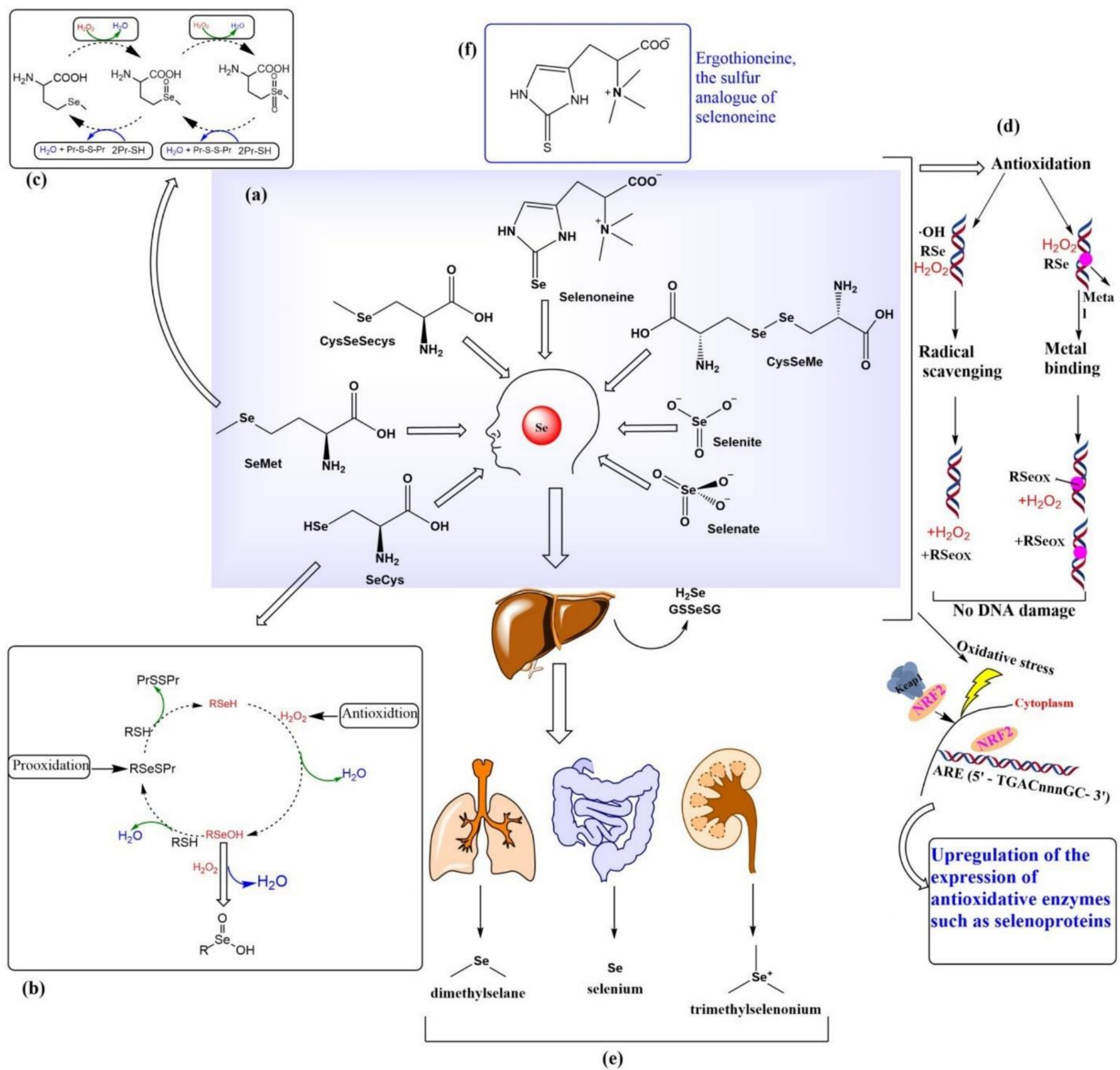


Figure 1: (a) A selection of naturally occurring selenium derivatives relevant for dietary intake. Among these compounds, selenocysteine and selenomethioneine exhibit the most pronounced biological activities (b) and (c) explain their catalytic cycles. (f) Insert: Ergothioneine, the sulfur analogue of selenoneine. (d) The mechanism of selenium antioxidants to prevent DNA damage by 1) reactive oxygen species (ROS) scavenging mechanisms, 2) the affinity of metal-bound to react with hydrogen peroxide to prevent the oxidation of metals, thus hydroxyl radical formation.

(e) Selenium excretion pathways

2. Reactive Selenium Species: Redox activity, catalysis, metal binding and more

The activity of selenium in biological systems is frequently associated with an underlying, pronounced redox activity which indeed is the hallmark of many Reactive Selenium Species (RSeS) under physiological conditions. This reactivity has given rise to the common conception that selenium is an "antioxidant" and hence may serve as a chemopreventive agent (18). This concept is, unfortunately, rather simplistic and naïve. It ignores the spectrum of different oxidation states of selenium in

biology, diverse redox mechanisms, catalysis and indirect cellular actions often triggered by such selenium compounds.

Figure 2 illustrates the Pourbaix diagram for Selenium, presenting the thermodynamically preferred oxidation states of selenium as a function of applied potential and pH.

Physiologically relevant oxidation states of this chalcogen range from +4 to -2 and hence we find highly reduced selenium species, such as H_2Se and organic selenols (RSeH),

which are indeed also highly reducing, on the one side, and oxidized species, such as SeO_3^{2-} and organic seleninic acids (RSe(O)OH), which are efficient oxidizing agents, on the other. At the same

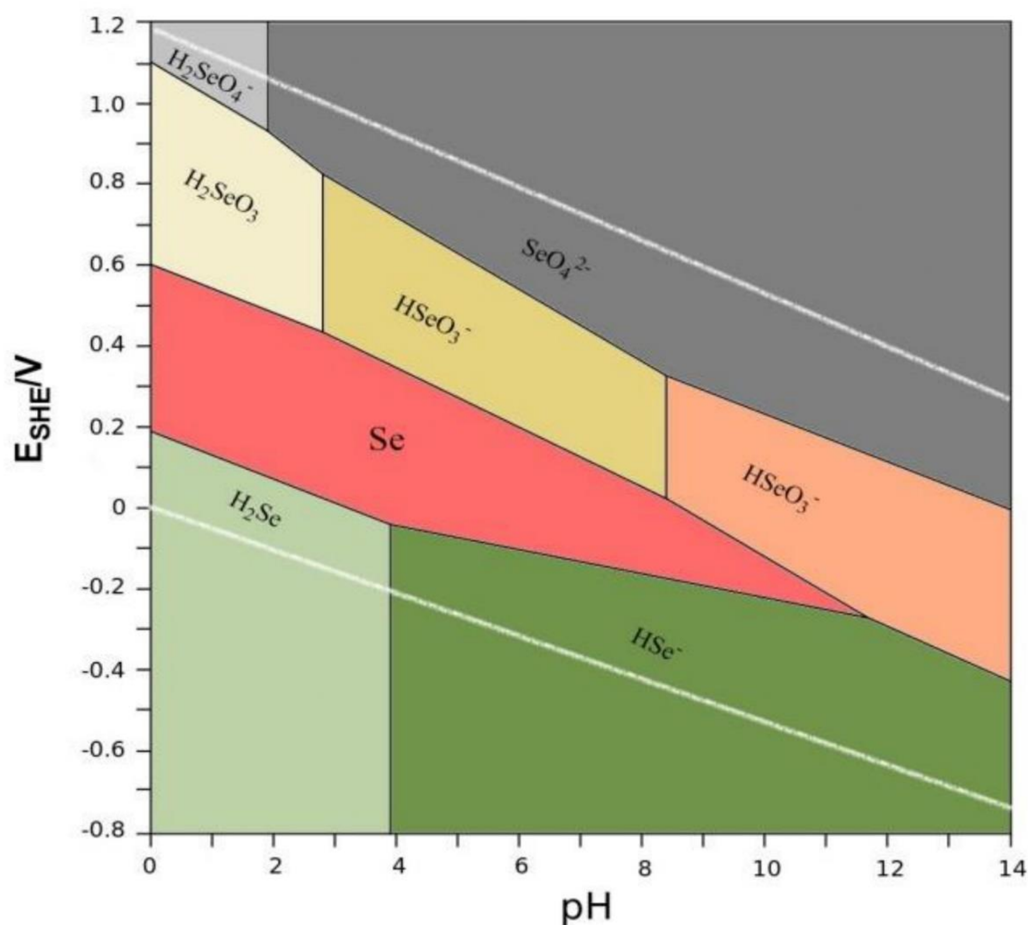


Figure 2: Pourbaix diagram of selenium at 25 °C. The white lines define the stability region of water

time, most selenium species hardly undergo simple one- or two-electron transfer reactions. Mechanistically, selenium rather acts as a nucleophile and hence via substitution and exchange mechanisms, and therefore has a certain preference for the

other chalcogens, *i.e.* oxygen and sulfur, as reaction partners, it can also be introduced as an electrophile or a radical, the electrophilic compounds (19, 20). These redox properties must be taken into consideration, and we will return to them later on.

In biology, matters are - literally - even more complex. Besides exhibiting a pronounced redox activity, which often provides the basis for catalysis, certain selenium species, such as selenol(ate)s, are also able to complex metal ions, including Fe^{2+}, Zn^{2+}, Cd^{2+} and Hg^{2+} (21, 22). This selenium complex chemistry has hardly been explored so far, still seems to be pivotal to our understanding of the biological activity of this element and its various compounds, as we will see when considering the case of selenoneine in more detail in section 3.

In addition to redox and metal binding activities, many selenium compounds also organoselenium compounds are strong reagents that act as intermediates, for. instance, the α-selenenylation of carbonyl

undergo catalysis or "decompose" with the release of biologically active selenium species - and each of these chemical reactivities may result in specific biological activities. This palette of reactivities is illustrated in Figure 1 for the two selenium-containing amino acids selenocysteine (SeCys) and selenomethionine (SeMet). Unlike SeCys, which as the 21st natural amino acids is incorporated into proteins *via* an eloquent mechanism involving an insertion sequence SECIS and "overwritten" stop codon UGA, SeMet can be incorporated randomly in place of methionine (23, 24). Once incorporated into proteins, these amino acids provide powerful centres for redox activity, catalysis, metal binding and also signalling in over 25 seleno proteins and enzymes found in humans, with a barrage of

other selenoproteins and enzymes present in animals, microorganisms and plants (25, 23). It is therefore not surprising that some of these enzymes have served as blue-prints for the design of small molecule selenium compounds mimicking some of their pronounced biological activities (26).

Moving on from basic chemistry to cellular networks, the issues surrounding selenium tend to become very complicated. Here, one often finds selenium agents at the centre of wider signalling cascades. At this stage, the various cellular events associated with these compounds are not merely the result of simple chemical interactions, they are rather "triggered" or controlled as part of wider - often antioxidant - cellular signalling cascades. It is therefore possible that - seemingly paradoxically - an oxidizing agent triggers an antioxidant response and *vice versa* (25). Furthermore, it has been observed that selenium supplementation in animals and humans not only increases selenium levels in the blood, it also initiates the expression of various selenoproteins and enzymes, including glutathione peroxidases (GPx), thioredoxin reductases (TrxRs) and the most common selenoprotein found in human blood plasma, *i.e.* Selenoprotein P (27). Here again, supplementation with a given selenium agent results in a wider antioxidant response, which is only indirectly due to the agent and its chemistry *per se*. The antioxidant behaviour associated with many selenium compounds therefore often results from indirect events, such as the initiation of the nuclear factor erythroid 2-related factor 2 (Nrf2) signalling pathway or an increase in the expression of GPx (28). These particular circumstances must be considered when the "best" or "most potent" selenium supplements or antioxidants are being discussed from a chemical point of view.

Interestingly, such a "discussion" has been ongoing since the discovery of the first selenoenzyme in humans, namely GPx, in 1957 by Gordon C. Mills, and initial attempts to mimic its antioxidant activity in the 1980s (29, 30). Since then, various attempts to identify selenium proteins and enzymes, first by tedious isolation, and, more recently, by mining the genome for the "selenocysteine insertion sequence" (SECIS) have seen a rising tally of seleno proteins and enzymes found in animals and humans, and of the synthetic mimics associated with them (31, 30).

It is therefore truly astonishing that at the same time, there are hardly any small molecule selenium compounds naturally found in our body, apart from the usual suspects, such as H_2Se and its "volatile" metabolic derivatives mono-, di- and trimethylselenide (CH_3SeH, $(CH_3)_2Se$ and $(CH_3)_3Se^+$, respectively), and a few other intermediates along the selenium pathway, such as selenodiglutathione (GSSeSG) (32). Even these examples are not terribly impressive from a chemical perspective, and as metabolic intermediates, are also not really the kind of "super antioxidants" one may expect to find in human physiology. On a low note, most of the derivatives of H_2Se mentioned here are actually "waste" products and prepared for excretion via the lungs or urine (33-35).

Paradoxically, this rather limited number of small molecule selenium compounds found naturally in the human body has not deterred researchers to dwell on synthetic selenium compounds as potent antioxidants or as promising agents in cancer treatment and prevention. The main limitation of small selenium compounds application is linked to the double-behaviour of selenium as an antioxidant and a prooxidant. For instance, selenocyanates, selenoureas, selenoesters,

selenium-containing heterocycles, and recently selenium nanoparticles are getting immense attention due to their significant bioavailability, in addition to selenides and diselenides (36, 37).

Over the years, a myriad of synthetic selenium compounds has been designed, developed, evaluated and explored, often mimicking the selenium-containing antioxidant enzymes GPx and thioredoxin reductases TrxRs (38). Ebselen, a synthetic organoselenium that was first studied for its GPx-like activity in the 80s, can catalyse the Nicotinamide Adenine Dinucleotide Phosphate Hydrogen (NADPH)-dependent reduction of hydroperoxides through the oxidation of glutathione (GSH) in glutathione disulfide (GSSG). In that way, Ebselen exhibits an antioxidant activity, by reducing peroxides using thiols as electron donors. (39-41). This organoselenium compound has been under intense scrutiny in clinical trials, first as antioxidant and, more recently, in neuroprotection and in the protection and treatment of cardiovascular diseases (42). Nonetheless, none of these compounds has so far reached a defined medical application as a certified medication. The field of "selenium drugs" has therefore long been met with a certain disinterest and even suspicion among the pharmaceutical community in general, and the respective industry, in particular.

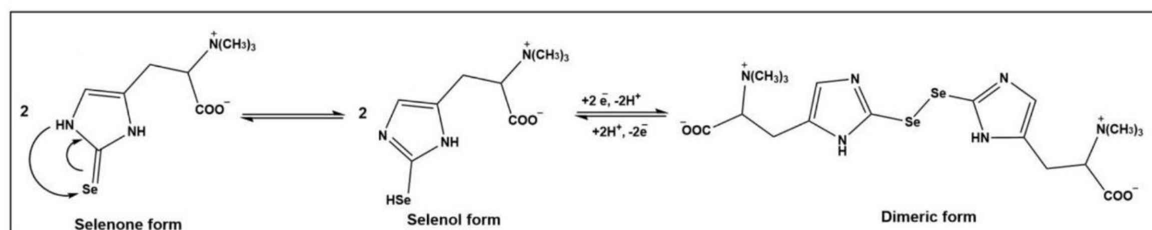
It has therefore been surprising, and also stimulating, when in 2010 Yamashita *et al.* identified selenoneine in the blood of blue tuna, *Thunnus orientalis* , at concentrations in the range of 5 - 40 μg Se/g (43•• -45••). Surprising, because of the otherwise notable absence of selenium compounds in animals. Stimulating, as selenoneine is not "just" a simple selenol or selenide, it rather undergoes an unique selenol-selenone tautomerism, which endows this simple yet elegant selenium compound with considerable reactivity and a range of amazing biological

activities, some of which are only just about to emerge these days.

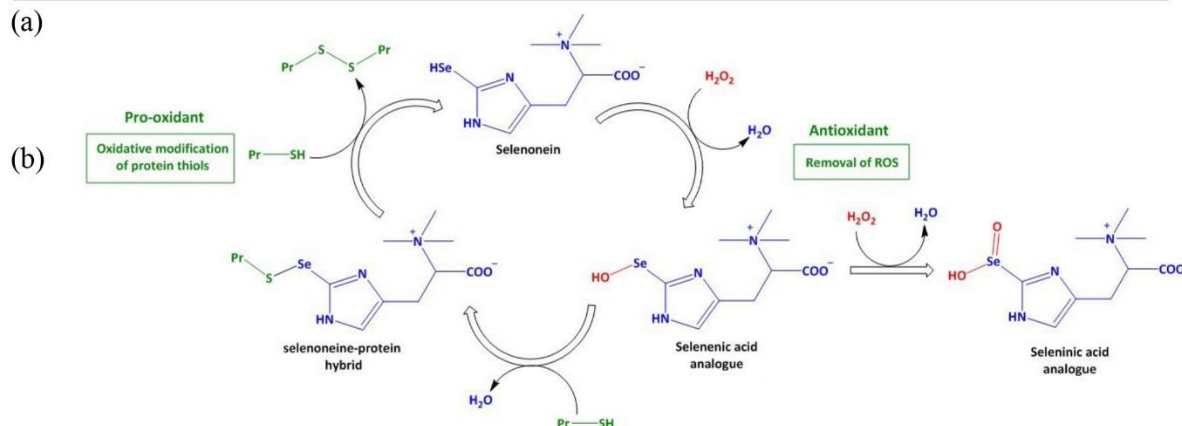
3. The selenol-selenone conundrum: Redox activity and metal binding

Selenoneine is the selenium analogue of ergothioneine (Figure1), a sulfur-containing derivative of the amino acid L-histidine, which was isolated from ergot in 1909 and is found in both, plants and animals. Mammals receive ergothioneine solely from their diet, for instance by consumption of mushrooms, dark beans and oats, which contain high levels of this Reactive Sulfur Species (46•). Ergothioneine has been studied extensively since its discovery, for its legendary radical scavenging activities, the considerable chemopreventive properties and the potential use in chronic immunodeficiency diseases (47-51).

Just as its more prominent sulfur analogue, selenoneine belongs to a particular - and particularly interesting - class of chemical compounds, which exhibits a specific kind of chalcogen-centred tautomerism, in this case the selenol-selenone tautomerism. This particular equilibrium is illustrated in Figure 3. It implies, among others, that selenoneine exists in two chemically distinct forms, which are also distinctively different in their chemical reactivity and may become oxidized to a more stable dimer. Under standard conditions of biological chemistry, the equilibrium resides on the side of the selenol, still it is pH dependent and one therefore needs to account for both forms. Whereas the selenol form represents an easily deprotonatable species that are weak acids similar to the traditional



(a)



(b)

Figure 3: (a) The two tautomers of selenoneine the selenone form and the selenol form and the stable oxidized dimer. (b) the catalytic cycle of selenoneine exhibiting antioxidant activity by removing ROS and pro-oxidant activity by the oxidative modification of protein thiols

selenolates, the selenone form, sometimes also referred to as selenoxo form to avoid confusion with the selenium [1] analogue of sulfones sailing under the same name, is more flexible in its reactivity, chemically somewhat resembling the behaviour of selenoureas (Figure 3).

This kind of two-faced chemistry of selenoneine sets the molecule apart from most of the other selenium compounds under investigation and is the cause for a pronounced biological activity.

To date, the literature on this topic is limited, still the few reports available so far point

[1] Unlike the corresponding sulfur derivatives, the sulfones, the selenones ($RSe(O)_2R'$) are equivocal to the selenones ($RC(=Se)R'$) in terminology but not in chemistry. These Reactive Selenium Species are not stable and are usually not found in Nature.

towards a considerable activity, in some instances orders of magnitude higher than the one associated with ergothioneine. Similar to ergothioneine, selenoneine is an excellent scavenger of free radicals, and the 50% radical scavenging concentration (RS_{50}) of selenoneine, as measured in the DPPH (1-diphenyl-2-picrylhydrazyl) assay, is just 1.9 µM, with L-ergothioneine at 1.7 mM and Trolox, for comparison, at 880 µM (45••). This amazing activity is vital for fish as far as adaption and survival in low oxygen marine environments is concerned (52). In human cell models, a similar antioxidant activity has been observed. In a very recent study by Seko *et al.*, for instance, 10 µM concentrations of selenoneine increased cell viability of cultured human leukemic K562 cells by 14.4% in the presence of 0.2 mM *tert*-butyl hydroperoxide (t-BHP), by 9.2% in the presence of 50 mM AAPH (2,2′-Azobis(2-methylpropionamidine) dihydrochloride) ,by 8.3% in the presence of 0.1 mM pyocyanin, and by 10.4% in the presence of 2 mM GSNO (*S*-Nitrosoglutathione), as compared to the selenoneine-free medium (53••).

Besides acting as radical scavenger, selenoneine is also able to protect cellular proteins more indirectly from oxidation, it is also important to mention that selenols are generally more active than thiols and are ionized at neutral pH, thus staying in anionic form at the catalytic site. Selenols have a samller p*K*a (7.5) than thiols (8.4) and they act as strong nucleophiles with low redox potential giving them more potential in redox catalysis. Selenoproteins protect against metal toxicity and have a significant antioxidant activity mainly attributed to the highly reactive selenol group (54-56). As mentioned already, reduced forms of selenium often are excellent ligands for soft metal ions, such as Fe^{2+} and Hg^{2+} - and

selenoneine is no exception. It binds strongly to the haem moiety of myoglobin and haemoglobin, hence protecting these central iron proteins from auto-oxidation, for instance under conditions of hypoxia, when excessive amounts of Reactive Oxygen Species (ROS) may be present inside the red blood cells (43••). Nonetheless, selenoneine is probably unable to protect all myoglobin molecules at the same time, as the concentration of selenoneine in purified myoglobin from bluefin tuna was estimated at a Se:Fe molar ratio of just three to 1,000 (44••).

For comparison, the Se:Hg molar ratio in fish was estimated to be 3:1 (43••, 44••, 57••). Indeed, the ability to bind to metal ions is also essential for the detoxification of Hg^{2+} ions, with selenoneine controlling the metabolism and removal of methylmercury (CH_3Hg^+) from blood circulation. The underlying mechanism has been demonstrated by Yamashita *et al.* in rather elegant models of human embryonic kidney HEK293 cells, and also in zebrafish, which were transiently overexpressing the OCTN1 (organic cation/carnitine transporter 1) which simultaneously acts as an ergothioneine transporter (58••). In these studies, the (- chemically more stable -) oxidized dimer of selenoneine reduced levels of ROS and enhanced cell proliferation. The transporter for ergothioneine, and *ergo* also for selenoneine, appears to be essential for this activity. In the OCTN1-transfected HEK293 cells, addition of selenoneine resulted in a decrease of mercury accumulation, whilst no such activity could be observed in the OCTN1 deficient counterparts. It is notable that selenoneine, just as ergothioneine, is able to enter cells via this unspecific organic cation/carnitine transporter. As we will see in section 5, such similarities between the two chalcogen analogues not only entitle selenoneine to enter various cells, they also

form the basis for the facile biosynthesis of this particular selenium metabolite in genetically engineered organisms.

The pronounced antioxidant and protective activity observed for selenoneine in these assays has encouraged additional cell based and *in vivo* studies. Several subsequent studies have reported a pronounced anticancer activity of selenoneine. Recently, Masuda *et al.* have reported a reduction of colorectal cancerous cells in mice (59••). In this study, a diet of selenoneine-containing tuna dark muscle extract (STDME) was employed to assess whether the antioxidant capacity of selenoneine could be sufficient to inhibit the development and growth of such tumours. Whilst a reduction of tumour development has indeed been observed, this activity might not necessarily be just due to the antioxidative properties of selenoneine. Indirect events, such as the inhibition of splenic Myeloid-derived suppressor cell (MDSC) accumulation, may also be responsible for this observation. MDSCs often inhibit innate and adaptive immunities *via* multiple mechanisms and accumulate during tumour growth resulting in extensive ROS production (60-63). As pointed out in section 2, such indirect events associated with a particular selenium compound may dominate the more direct chemical reactivity.

As selenoneine is a comparably "novel" natural product on the scene, the investigations of this unique selenium compound are still limited. This is also due to the fact that selenoneine is not readily available commercially, the isolation from biological samples is cumbersome and the chemical synthesis of selenoneine involves multiple steps. The range of biological activities and the underlying mode(s) of action are therefore not fully explored yet and may carry some surprises. Indeed, a preliminary analysis of the antioxidative

effects of selenoneine on human leukemic K562 cells by Seko *et al.* suggests that selenoneine may actually not act in isolation, it may rather react with cysteine residues in proteins, subsequently forming protein complexes with high molecular weight and with antioxidant activity (53••). Similar ideas of "piggy backing" selenium onto initially selenium-free cellular proteins have been around for a while, and this may be a natural confirmation of the medical value of these approaches (64).

4. Fisherman's friends

One of the most notable health benefits of selenoneine may be the protection of erythrocytes, either directly by scavenging radicals or indirectly, by binding to and hence protecting iron proteins, including haemoglobin. Indeed, persons with a diet rich in tuna fish also tend to exhibit higher concentrations of selenoneine in their erythrocytes of 0.212 μg selenoneine Se/g, which may provide an additional protection from oxidative stress. A study by Yamashita *et al* 2013 involving 167 participants inhabiting a remote Japanese island has confirmed that selenoneine is the major form of selenium in red blood cells, with its presence related closely to the intake of sea fish. As one outcome of this study, the authors have noted that the concentration of selenoneine in the cellular fraction of human blood may actually be a biomarker for fish-derived selenium in the human body (57••). Various other studies have confirmed the interplay between a diet rich in fish on the one side and selenium and selenoneine levels in the human body on the other. The selenium concentrations found in red blood cells in general correlate with the dietary intake of selenium-containing food, such as seafood, and the presence of selenoneine, in particular, is linked to consumption of fish (65•• -69).

Among the various fishes and dishes, tuna appears to be particularly rich in selenoneine, with several times lower concentrations found in other animals, such as squid, tilapia, pigs and chicken (45••, 43••). The red muscles of tuna contain around 190 nmol Se/g selenoneine, and just 4.5 nmol Se/g selenoproteins. Approximately 98% of the organic selenium in the muscles of this fish is therefore present as selenoneine. The blood of tuna and mackerels shows even higher values of selenoneine, in the range of 430 to 440 nmol Se/g. Notably, selenoneine is also distributed in other tissues of tuna, it is found in the heart, spleen and white muscles, although at lower concentrations of around 12 nmol Se/g. Apart from tuna, selenoneine is present in the blood of tilapia fish, the heart and liver of chicken, and the kidneys of pigs (44••).

If one moves to the tune of selenoneine for the protection of red blood cells, tuna is the best choice, also because of bioavailability. Selenium derived from fish shows excellent bioavailability, as confirmed already several decades ago in a study by Hagmar *et al.* (46•). This study indicated that the levels of selenium were around 80% higher in fishermen with a diet rich in fish, *i.e.* 21-50 fish meals/month, when compared to a control group with just 0-3 fish meals/month. Notably, in addition to its protective activity, selenoneine appears to be non-toxic to humans, quite in contrast to some of the other seleno-compounds under discussion as food supplements, such as selenocysteine with a LD_{50} of 35.8 mg/kg, selenomethionine with a LD_{50} of 4.3 mg/kg or selenite with a LD_{50} of 3.5 mg/kg (44••). Selenoneine may therefore also be considered as a valuable source of nutritional selenium, regardless of any of its other potentially beneficial biological activities.

5. Sources of selenoneine

In the context of nutrition, tuna fish is obviously the prime natural source of selenoneine. Still, the concentrations contained in fish are rather low, and obviously insufficient to cover the demand, especially if and when selenoneine may be considered as a healthy food supplement. Indeed, whilst selenoneine derived from fish may represent an attractive source of selenium for Captain Birdseye and team, this approach is not viable ecologically and economically on a larger scale - and also not particularly tuna- or vegetarian-friendly. At the same time, the biosynthesis of selenoneine in tuna fish is still largely unexplored.

Interestingly, biotechnology has recently opened up an alternative avenue, whereby certain fungi and bacteria have been manipulated genetically in order to produce selenoneine. Genetically engineered *Schizosaccharomyces pombe* fed on selenate (Na_2SeO_4), for instance, is able to produce around 700 mg of oxidized selenoneine per one litre of original culture (70••, 71••).

Such biotechnological approaches are interesting from the perspective of food and cosmetic industries, where "biologically produced" selenoneine is required to satisfy the demand of consumers. In research and drug development, the chemical synthesis of this chiral derivative of histidine represents a promising alternative. A patent by Li Wei, Liu Yuanyuan, Xiong Xiaohui, Li Fangshi

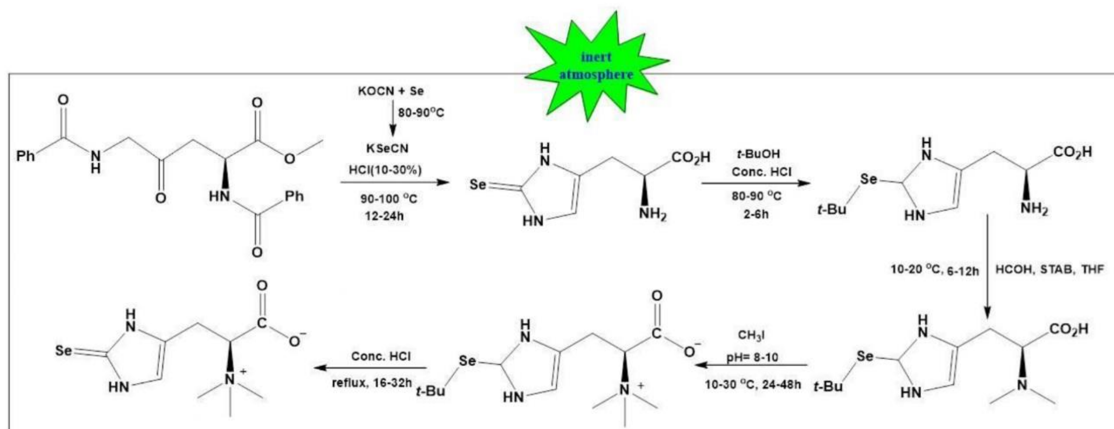


Figure 4: Chemical synthesis of selenoneine as described in a recent patent by by Li Wei, Liu Yuanyuan, Xiong Xiaohui, Li Fangshi iu and Wei Ping (application CN104072423A) (72••).

and Wei Ping (application CN104072423A) has reported the only developed multi-step synthesis of selenoneine in 2014 which is distinctively different from the one for the sulfur-analogue ergothioneine. Depicted in Figure 4, this multi-step synthesis commences from N2,N5-dibenzoyl-4-oxo-L-ornithine methyl ester and potassium selenocyanate (KSeCN) as donor of selenium. It involves several condensation, cyclisation, protection, deprotection and methylating steps and is attractive due to the simplicity of most of these steps, the mild reaction conditions, readily available reagents and also an impressive yield of 55 % at the last step (72••).

6. Conclusions

Selenoneine has been reported less than ten years ago. Despite this comparably short period of time on the selenium research menu, it has already raised considerable interest, from synthetic and redox chemistry to biology, nutrition, cosmetics and drug development. The selenium analogue of ergothioneine is indeed very special. Found

primarily yet not exclusively in tuna fish, it employs cellular transporters designed for organic cations and carnitine to enter human cells, in particular red blood cells, where it serves as antioxidant and protector of haem groups.

Thanks to its fairly unique selenol-selenone tautomerism, selenoneine not only scavenges radicals, it also binds to metal ions and, similar to ergothioneine, has the potential to "turn catalytic" and may even attach itself to proteins, forming specific selenoneine-protein hybrids as it is shown in Figure 3.

Today, biotechnology and synthetic chemistry both promise wider access to selenoneine and selenoneine research. This will enable researchers to investigate the full spectrum of biological activities and underlying mode(s) of action of this compound in considerable detail. Such studies will also determine if selenoneine may be employed as nutritional supplement, for instance in the elderly, and which benefits such an alimentation may, or may not, provide. With the chemical synthesis of selenoneine now established, it will also be possible to produce a range of - equally interesting - derivatives. At the same time, and inspired by the analogy between selenoneine and ergothioneine, additional small molecule selenium agents may emerge from and in animals by moving from the established (46•) into the realm of its chalcogen neighbour. Ovothiols, for instance, represent a group of highly interesting natural sulfur metabolites, and one may wonder if there are any "selenovo"-style selenium analogues of these substances hidden somewhere in nature.

In any case, the discovery of selenoneine has provided new stimulus to selenium research, from the hunt for natural selenium

metabolites and nutrition to synthetic chemistry and biology.

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Compliance with Ethical Standards

Conflict of Interest

There are no real or potential conflicts to declare

Human and Animal Rights and Informed

All reported studies/experiments with human or animal subjects performed by the authors have been previously published and complied with all applicable ethical standards

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1.7 Unveiling selenium: techniques for quantification and specification

As already mentioned, selenium can only be obtained through our diet. Therefore, identifying and quantifying selenium species in various food products and in the soil is essential to ensure adequate intake of selenium. However, measuring selenium content in different food matrices has traditionally posed challenges due to its very low concentration in most foods varying with food sources and culture location and the complexity of food matrices with the presence of numerous compounds such as fats, carbohydrates, vitamins. Therefore, a myriad of analytical methods and techniques have been developed to overcome these challenges. Today, scientists use advanced approaches to measure selenium concentrations in food and soil samples and aim to provide accurate and reliable data for dietary and nutritional assessments.

Article III: **Analytical Methods for the Quantification of Selenium Species in Biological Matrix: Where are We?**

Rama Alhasan, Caroline Perrin-Sarrado, Claus Jacob and Caroline Gaucher

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Objective: Over the years, scientific research has demonstrated the importance of selenium as an essential element for mammals, highlighting its activity against numerous diseases and also its prophylactic effects. It has also been shown that an insufficient intake of selenium can be harmful. Consequently, the nature and concentration of selenium and its derivatives in the diet, in the body and in the environment, such as soil, need to be carefully determined.

Methods: This review summarises analytical methods for the identification and quantification of selenium in biological samples.

Results: Routine to advanced methods are reviewed, focusing on their analytical characteristics such as specificity for different types of selenium, sensitivity, precision, reproducibility and skill requirements.

Conclusions: There are already many studies on the analysis of selenium species. In addition to the quantification method used, we propose to review the pre-analytical steps involved in the handling of biological samples, which directly affect the results, and whose accuracy can be improved by careful pre-treatment. In addition, different combinations of techniques may be useful to obtain better results in terms of selenium species identification. Here we review the latest state-of-the-art methods for the identification and quantification of selenium, such as high-performance liquid chromatography coupled to inductively coupled plasma mass spectrometry (HPLC-ICP-MS), hydride generation atomic absorption spectrometry (HG-AAS), hydride generation atomic fluorescence spectrometry (HG-AFS) or inductively coupled plasma optical emission spectrometry (HG-ICP-OES). This study highlights the importance of this research and the need to achieve reliable, safe and effective quantification and determination methods.

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MINI-REVIEW ARTICLE

Analytical Methods for the Quantification of Selenium Species in Biological Matrix: Where are We?

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Abstract: ***Objective***: Over the years, scientific investigations have proven the importance of selenium as an essential element for mammals, emphasizing its activity against many diseases and even its prophylactic effects. It is also established now that a malconsumption of selenium can be harmful. Therefore, the nature and the concentration of selenium and its derivatives found in the diet, the body, and even in the environment, for example, in the soil, should be determined carefully.

Methods: In this review, analytical methods for speciation and determination of selenium concentrations in biological samples are summarized.

Results: Methods ranging from routine to cutting-edge are explored, focusing on their analytical characteristics, such as specificity for discrete selenium species, sensitivity, accuracy, reproducibility, and skills required.

Conclusion: There are already numerous studies regarding the analysis of selenium species. Beyond the method employed for actual measurements, we propose to review the preanalytic steps for sample handling in biological matrices, which directly affect results that will be more accurate with careful pretreatment. Furthermore, to reach better outcomes in terms of the identification of selenium species, different combinations of techniques might be the answer. We highlight here the last and the cutting-edge methods to identify and quantify selenium such as, high-performance liquid chromatography combined to inductively coupled plasma mass spectrometry (HPLC-ICP-MS), hydride generation atomic absorption spectrometry (HG-AAS), hydride-generation combined to atomic fluorescence spectrometry (HG-AFS), or to inductively coupled plasma optical emission spectrometry (HG-ICP-OES). This review emphasizes the importance of such investigations and the need to achieve reliable, safe, and effective quantification and methods of determination.

Keywords: Analysis, biological samples, essential elements, health benefits, matrices, selenium.

1. INTRODUCTION

Selenium (Se), a chalcogen element, was discovered in the early 19th century in Sweden. It is naturally present in the soil in highly variable contents ranging from 5 to 1200 000 µg/kg. It is taken up by plants and transferred to higher trophic levels through the food chain. Therefore, selenium content in food reflects the available selenium content of soils used to cultivate those plants [1]. The main source of selenium for humans is their diet (Fig. **1**), with selenium concentrations varying between different types of food [2-5]. Notably, the concentration of selenium in these items may vary considerably depending on the soil and the method of cultivation. Please note that drinking water itself hardly contains selenium, which should be limited to 10 µg/L according to the French national agency for food, environmental and work safety, 30 µg/L according to the US office of environmental health hazard assessment or 40 µg/L, according to the WHO (2011).

As a trace element, selenium is important for mammals in general and humans in particular, albeit its role has only been discovered, in 1957, by the German biochemist Klaus Schwarz, who proved its importance for mammalian wellbeing [6]. In fact, selenium plays a role in various pathophysiological processes such as physiological development, boosting immunity, protection from cardiovascular diseases, redox modulation *via* redox enzymes, such as the glutathione peroxidase (GPx) family, and possibly also prevention of various types of cancer [7]. Indeed, various studies have confirmed that selenium deficiency may result in the development of cancer, cardiovascular diseases, rheumatoid arthritis, cardiomyopathies, and osteochondropathies, such as

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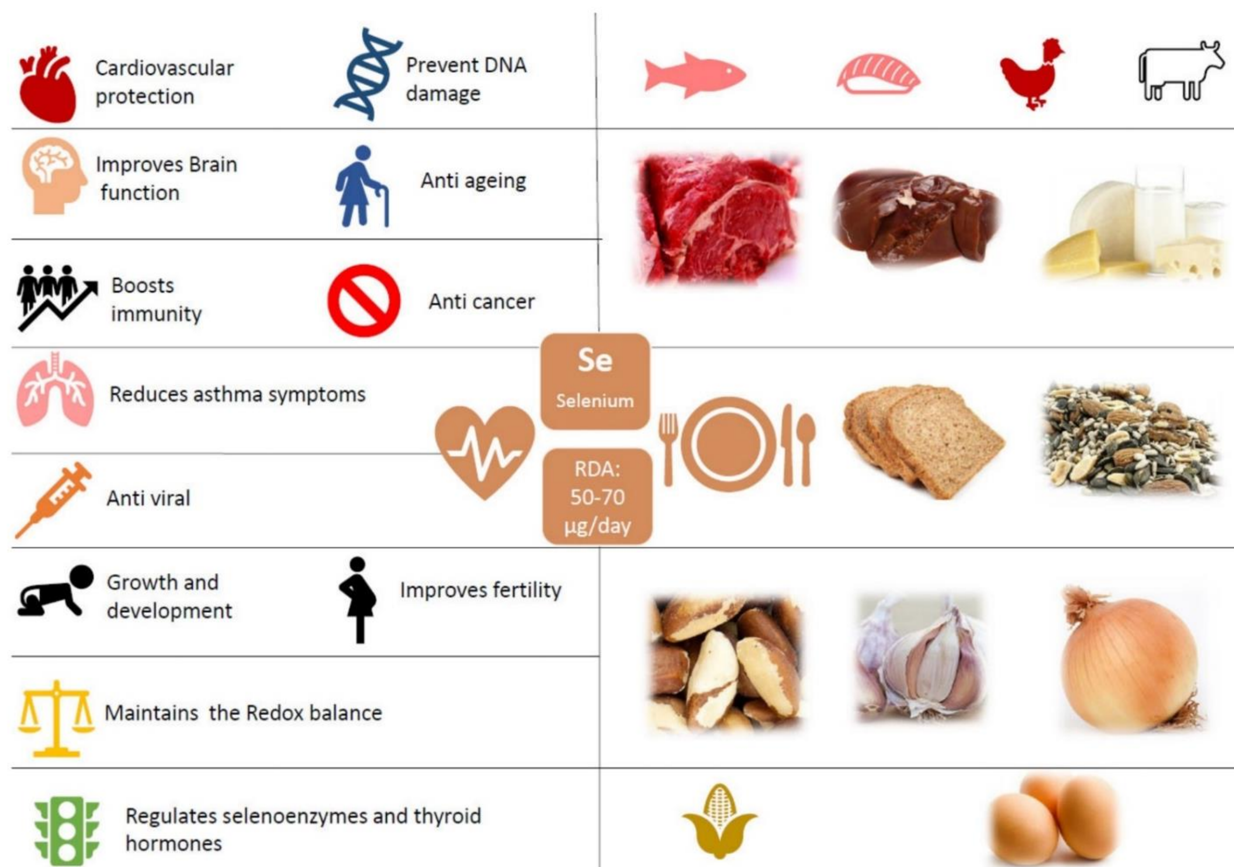


Fig. (1). Dietary sources and health benefits associated with and assigned to selenium (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

Keshan and Kashin-Beck diseases [8-16]. As for most redox-active compounds, selenium has a dual nature depending on its concentration, *i.e.,* antioxidant at low concentration and prooxidant at high concentration. These important health benefits are illustrated in (Fig. **1**).

Therefore, excessive supplementation of selenium is equally undesirable, and the maximum daily dose of selenium for adult humans is 55-70 µg per day with a maximum tolerated amount of 400 µg per day. Indeed, higher doses may cause selenosis, which is associated with nausea, diarrhea, garlic breath, loss of hair and nails, metabolic acidosis, and impaired activity of the gastrointestinal and nervous systems [17, 18]. Therefore, the dose used in experimental studies to boost immunity is 100 µg per day [19-21]. As for its redox activity in organisms, selenium at its recommended dietary allowance acts as an antioxidant to prevent oxidative stress (OS)-related pathologies, and at a high dosage, up to 1600 µg per day, selenium compounds are employed to induce ROS production and initiate OS-related death of various cancer cells [22, 23].

Moreover, selenium tolerance depends on its chemical form, *i.e.,* organic forms are typically better tolerated by mammals at higher concentrations than inorganic forms [23-25]. Furthermore, mammals are not able to synthesize seleno-amino acids such as selenomethionine (SeMet), whereas plants, yeast, and some edible mushrooms can. For instance, plants take up selenite (SeO_3^{2-}) and selenate (SeO_4^{2-}) from the soil and turn them into different forms of selenium by a sequence of biochemical events, mostly *via* PO_4^{3-} transporters and SO_4^{2-} transporters, and *via* the reduction to selenides by glutathione. Selenide interacts afterward with *O*-acetylserine forming selenocysteine (SeCys), which finally results in the biosynthesis of SeMet *via* methionine synthase [26, 27].

In yeast, the accidental biosynthesis of SeMet from SeO_4^{2-} or SeO_3^{2-} takes the same path as in plants. Moreover, yeast can take up SeMet from external sources. The incorporation into proteins is usually nonspecific as enzymes are unable to differentiate between methionine (Met) and SeMet. This allows SeMet to mistakenly take on the place of its sulfur analog within proteins [27].

In mammals, selenium is the only essential element incorporated into proteins during mRNA translation, in the

form of special amino acids, such as SeMet and SeCys, which can be incorporated into proteins in the place of Met and cysteine (Cys), producing proteins containing seleno-amino acid. The biological activities of selenium are mainly attributed to the insertion of SeCys into several key proteins, such as the GPx family, thioredoxin reductase, and iodothyronine-5 deiodinases exhibiting enzymatic activity [12, 28, 29]. Notably, SeCys is the 21^rst amino acid encoded by the UGA codon, which is usually recognized as a stop codon. Thus, it requires a special process for its incorporation into proteins. This process involves a selenocysteine tRNA (Sec-tRNA$^{[Ser]Sec}$) and a selenocysteine insertion sequence (SECIS). Then, SeMet needs to be converted to SeCys *via* the *trans*-selenation pathway similar to the *trans*-sulfuration pathway for Met synthesis. Furthermore, the sulfur-containing amino acid Met and SeMet cannot be distinguished by a Met-tRNA. Therefore, SeMet is incorporated into proteins nonspecifically, increasing protein antioxidant activities [30-34]. Indeed, although selenium shares many similarities with sulfur, selenocompounds have lower *pKa* values, better electrophilicity, nucleophilicity, and better leaving groups [35]. Selenium is also a good ligand for various metal ions and here it binds, for example, arsenic, cadmium, and mercury, often decreasing their toxic impact on organisms [8, 28].

The variations in the distribution of selenium content and species in plant and animal-based foodstuffs Table **1** depend on different environmental conditions, in particular the quantity and species of selenium to which the plant or animal are exposed to [36, 37]. Interestingly, the form of selenium found in foodstuffs may also vary. SeMet, for example, is the dominant form in cereals [9]. The major forms of selenium in other types of foods are SeO_4^{2-} and SeMet, plus low concentrations of SeCys. The major form of selenium in selenium-accumulating plants is γ-glutamyl-Se-methylselenocysteine [38, 39]. Data concerning the forms of selenium found in animal-based food is limited, and it looks as if the major forms are SeMet and SeCys incorporating nonspecifically into muscle proteins [40]. In addition, SeO_3^{2-} and selenoneine have been detected in fish [8, 41, 42].

Although the consumption of specific nutritional items rich in selenium may cover some of the daily required amounts, an intake of selenium fortified foods or dietary supplements is notably increasing nowadays. Such fortification is, in principle, possible *via* two distinct avenues, either by enriching and fortifying plants naturally *via* selenium (*i.e.*, sodium selenate) containing fertilizers, such as AgSel™, or by artificial supplementation with traditional tablet or capsule style supplements. Such selenium-containing supplements mostly include inorganic selenium forms, such as SeO_3^{2-} and SeO_4^{2-} ions, and organic selenium forms, such as SeMet, SeCys, and methylselenocysteine (MSC), and the selenium-enriched *Saccharomyces cerevisiae* yeast (SeY) [8, 43]. SeY contains a mixture of selenides and is widely used due to the fast cultivation of yeast, simple culture conditions, significant selenium accumulation, and high bioavailability of selenium [44].

Table 1. A brief selection of common food items and their respective selenium content.

Source	Selenium Content µg/g
Brazil nuts	0.2 - 512.0
Cereals	0.01 to 0.55
Dairy products	0.1
Eggs	0.09 - 0.25
Fish	0.06 - 1.50
Fish such as tuna	0.7 - 1.0
Fruits and vegetables	0.10
Garlic	0.4 - 4.0
Liver, kidney	0.4 - 1.5
Muscle meats	0.104
Onions	0.5
Tap water	10 to 40 µg/L maximum tolerated depending on country regulations

As selenium supplementation needs to be controlled precisely, quantification of this trace element in foodstuffs is of utmost importance. Unfortunately, the quantification of trace amounts of trace elements is a challenging task for analytical chemists, especially when this trace element exists in so many different forms in samples of a compound nature. Indeed, there are 25 selenoprotein genes in humans identified so far, which encode selenoproteins with various activities, including selenoproteins P and W and the GPx 1, 3, and 4, which collectively have been assessed as biomarkers of selenium status. The most promising biomarker among them appears to be selenoprotein P, which typically represents approximately half of the selenium content of plasma and peaks after 2-4 weeks of selenium supplementation [45-49]. As selenium exists in different organic and inorganic forms, establishing a full selenium card evaluation is quite challenging. In many cases, pretreatment of the matrix is required, and the selection of the preanalytic treatment depends on matrix characteristics, selenium species and the analytic method selected for the separation and identification. For instance, aqueous leaching and enzymatic or basic/acidic sample digestion have been employed for the extraction of seleno-amino acids. Many routine and reliable detection methods are now available for the analysis and the determination of selenium content, such as neutron activation analysis, spectrophotometry, spectrofluorimetry, electrothermal atomic absorption spectrometry, high performance liquid chromatography-inductively coupled plasma mass spectrometry (HPLC-ICP-MS), gas chromatography (GC), polarography, hydride generator atomic absorption spectroscopy (HG-AAS), and graphite furnace atomic absorption spectroscopy (ET-AAS) [50-57]. Nonetheless,these methods require sophisticated and expensive instrumentation and often have limited sensitivity. This review will shed light on some of the widely employed methods, highlighting their strengths and drawbacks.

2. ANALYSIS

2.1. Preanalytical Phase

Speciation of selenium involving the identification and the quantification of selenium species is very complex and requires careful preanalytical steps, such as selenium extraction from the matrix, its preconcentration and its derivatization. These steps will influence the accuracy and the quality of species separation and identification.

As already discussed, selenium speciation is a complicated process because selenium is found in food and biological samples at very low concentrations and in different forms. Thus, selenium species must be isolated and extracted quantitatively from the matrices without changing the original nature of the form. Afterwards, selenium species have to be identified and quantified correctly.

Moreover, preanalytical extraction of selenium compounds is generally based on enzymatic hydrolysis by multiple proteolytic digestions with proteases or basic/acidic digestion of the matrix with hydrochloric acid or methanesulfonic acid under reflux. Furthermore, methods based on enzymatic hydrolysis are employed at mild pH and temperature conditions, which minimize degradation, interconversion and volatilization of selenium compounds. Such methods are tedious as they often take several hours. Therefore, physical extraction using ultrasound energy, microwave irradiation or pressurization may help to reduce the period needed for extraction from hours to few minutes. Indeed, these techniques speed up matrix hydrolysis by disrupting the cell membranes and accelerating enzymatic action. For example, ultrasound-assisted enzymatic hydrolysis has been previously and successfully employed to quicken the extraction procedure of organometallic species in biological samples [58].

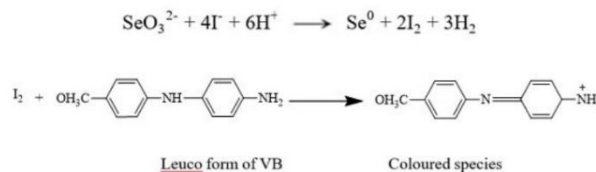
2.2. Colorimetric and Photometric Methods for Quantification of Selenium

2.2.1. Spectrophotometric Methods

Many analytical methods have been developed to determine the concentration of selenium in food, soil, and biological samples. Among the common methods are the ones depending on spectrophotometric analysis, using different chromogenic agents, such as 3,3-diaminobenzidine, chromotropic acid, 8-hydroxyquinoline, j-acid, 1-naphtylamine-7-sulfonic acid, Variamine Blue (VB), dithizone, and Leuco Crystal Violet. Unfortunately, some of these agents are either carcinogenic or have low sensitivity.

A rapid, simple, and sensitive spectrophotometric method depending on VB as colorimetric reagent has been developed to determine trace amounts of SeO_3^{2-} ions. This method is based on the reaction between SeO_3^{2-} (selenium IV) with potassium iodide (KI) in an acidic medium to liberate iodine, which oxidizes Variamine Blue forming a violet-colored molecule absorbing at 546 nm [59].

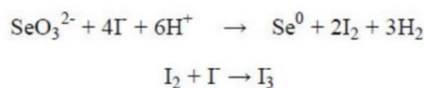
The reagent has a negligible absorbance at the same wavelength. The presence of ions such as Fe^{2+}, Sn^{2+}, CrO_4^{2-}, SO_3^{2-}, IO_3^-, VO_4^{3-}, Fe^{3+}, Cu^{2+}, Mn^{2+}, Ce^{4+} can interfere with the quantification method, thus, appropriate amounts of EDTA should be added to the solution.



The quantification method is linear from 2 µg to 20 µg of selenium in a volume of 10 mL. Molar and specific absorptivities of the violet-colored product and Sandell's sensitivity ($\mu g/cm^2/0.001$ Abs unit), which is defined as the lowest weight of the substance that can be detected in a column of the unit cross-section were found to be 2.6×10^4 L/-mol.cm, 0.33 $mL/g^{-1}.cm^{-1}$ and 0.003 $\mu g/cm^2$, respectively. The proposed method has high precision and has been applied successfully to the determination of selenium in water, soil, plants, human hair, and in cosmetics and pharmaceutical preparations [59].

A similar method applies iodine oxidation of *N,N*-diethyl-*p*-phenylenediamine dihydrochloride, resulting in a bright red compound with maximum absorption at 552 nm. Iodine is generated first by the reaction of SeO_3^{2-} ions with KI in an acidic medium. This analytic method provides high sensitivity, with a limit of detection (LOD) between 0.1 µg/mL and 0.2 µg/mL and linearity from 0.5 µg/mL to 3.0 µg/mL. Furthermore, this method shows high precision (RSD = 1.50%), accuracy (mean recovery = 99.96%), repeatability (RSD = 1.32%), simplicity and fastness to determine Se (IV) in selenium tablets, drinking water and cosmetic samples, such as in lipstick [60].

A rapid and less complicated spectrophotometric determination of SeO_3^{2-} ions using directly triiodide anions (I_3^-) without any chromogenic agent has also been developed. The analysis is based on the direct oxidation of iodide by SeO_3^{2-} ions in an acidic medium leading to the formation of elemental iodine. Elemental iodine reacts with excess iodide to form triiodide anions, which is the most stable soluble form in an aqueous solution. I_3^- color varies from yellow to brown, depending on its concentration with maximum absorption at 290 nm. Selenium detection ranges from 0.025 µg/mL to 0.100 µg/mL improving significantly the sensitivity of selenium quantification methods [61].



Even though spectrophotometric methods are commonly used and easy to perform, they still lack accuracy and do not respond to the diversity of selenium species to analyze, and to the diversity of complex matrices.

2.2.2. Fluorometric Methods

To detect and determine inorganic selenium, fluorescent reagents such as *o*-diamine, 3,3-diaminobenzidine dithizone, *o*-phenylenediamine, 2,3-diaminonaphthalene, Chrome Azurol S, and 8-hydroxyquinoline are commonly employed [51, 52, 62-66].

The following method is a simple and accurate on-line green method, which depends on the quenching effect of SeO_3^{2-} ions on Chrome azurol S fluorescence (λ_{ex} = 300 nm, λ_{em} = 407 nm) in the presence of sodium cholate bile salt (NaC). Today, this method is considered the gold standard for selenium quantification. The quenching mechanism is of a collisional type with a Stern Volmer constant value of 3.0×10^7 mol/L. Ions such as Cd^{2+} and Fe^{3+} interfere with the quantification of SeO_3^{2-} and this interference can be avoided using the chelation capacity of EDTA. To enhance outcomes and to reduce the volume of reagents and waste, an on-line configuration is proposed. Such a flow injection method exhibits a high rate of analysis, alongside a reduction of cost and waste. Under optimized experimental conditions, the LOD is around 0.27 µg/L with a linear range of concentration from 0.84 µg/L to 6.00 µg/L for SeO_3^{2-}. Furthermore, the chemical interaction with Chrome azurol S is selective for SeO_3^{2-} ions, without any affinity for SeO_4^{2-} ions (selenium VI). This methodology was employed successfully to analyze bulbous vegetables and biological samples with an average recovery for selenium close to 100% [64].

Another fluorescent method which quantifies nanograms of selenium in biological samples uses 2,3-diaminonaphthalene (DAN). This reagent reacts specifically with selenous acid (H_2SeO_3) in cyclohexane solution at 50°C for 20 min to produce 4,5 benzopiazselenol, which emits fluorescence at 521 nm after excitation at 379 nm. A linear relationship between fluorescence and selenium concentration is recorded up to 100 ng of selenium. Furthermore, the experimental scattering is less than 4% for selenium concentrations from 5 ng/mL to 25 ng/mL. The acidic digestion method using a mixture (2:1,v/v) of concentrated nitric acid (61%) and concentrated perchloric acid (70%) does not influence the linear relationship with the fluorescence intensity, as proven by a 99 ± 5% recovery of selenium concentrations from 2.5 ng/mL to 5.0 ng/mL added to digested or undigested samples. This method combines success, robustness, and simplicity, especially for biological samples from plants or animals containing small quantities of the trace element. Notably, this study was performed on muscles of tuna [67].

Those methods are selective for certain selenium forms, such as SeO_3^{2-}. Therefore, other types of selenium cannot be detected.

2.3. Voltammetric Techniques

Voltammetric techniques are established for specific measurements, often tend to be straightforward, sensitive, inexpensive, and may also be employed for speciation analysis in practice [68]. The Voltammetric technique described in what follows is a simple and accurate method for the separation and determination of SeO_3^{2-} ions, SeCys, and dimethyldiselenide. The method utilises differential pulse cathodic stripping voltammetry (DPCSV) on a hanging mercury drop electrode (HMDE) [69].

To employ this method accurately, samples need to be homogenized and leached under acidic conditions for 20-24 h at 4 °C in the dark. Then, the mixture is centrifuged and the supernatant is filtered and washed with HCl. The filtrate is analyzed directly by DPCSV IV. SeCys and SeO_3^{2-} ions are specified in the aqueous phase using HCl as an electrolyte. If employed successfully, the detection limits for SeO_3^{2-} ions and SeCys are 0.06 ng/mL and 2.00 Se ng/mL, respectively. Dimethyldiselenide can also be determined in the organic phase by adding $LiClO_4$ in absolute ethanol acidified with HCl as an electrolyte [69].

It is worth mentioning that the presence of sulfur-containing cystine in the sample, even at high concentrations, has no significant impact on the determination of SeCys, probably since disulfides and diselenides show distinctively different electrochemical potentials. This voltammetric technique has been optimised regarding parameters such as deposition time, voltage, and optimal acidity, extraction of the sample and effective separation of the Se species in aqueous and organic phases [69].

2.4. Separation and Identification of Organic and Inorganic Forms of Selenium

High-performance liquid chromatography (HPLC) is a very efficient separation technique and comprises different modes of separation, *i.e.,* reverse phase, anion exchange, and size exclusion, coupled with suitable detection systems, such as absorbance or fluorescence detectors, mass spectrometer (MS), or inductively coupled plasma mass spectrometry (ICP-MS). HPLC can be applied to nonvolatile samples with a wide range of molecular weights.

In 2019, a simple and sensitive HPLC method was reported to quantify selenocyanate ($SeCN^-$) in biological matrices, such as blood and cells. The method is inspired by the König reaction, which is usually employed for the quantification of cyanide and thiocyanate [70-72]. Notably, the preanalytical phase is crucial for this analytical method. Therefore, blood samples should be heparinized, diluted, and treated to accomplish the removal of proteins. Samples of cells are suspended in potassium phosphate buffer and homogenized. After centrifugation, the supernatant is basified with bicarbonate buffer and injected into the HPLC. Samples are analyzed by a post-column HPLC system coupled with a sensitive method of detection based on the König reaction, employing chloramine T aqueous solution as the chlorination reagent, which reacts with $SeCN^-$. A pyridine and barbituric acid mixture is added as fluorogenic reagent. Accuracy, sensitivity, and linearity are validated from 1 pmol/mL to 100 pmol/mL of $SeCN^-$ [73]. The LOD calculated and the limit of quantification (LOQ) are reported to be extraordinarily small, just 73.5 fmol/mL and 245.1 fmol/mL, respectively.

The HPLC method established for $SeCN^-$ fluorescent detection is also highly selective as the König reaction provides relevant specificity. This HPLC method is also sensitive and simple in comparison with previous photometric methods, as it allows the quantification of $SeCN^-$ at low concentrations in a biological matrix [73].

Related approaches towards the quantification of trace amounts of selenium are focusing on HPLC or gas chromatography (GC) conjoint with inductively coupled plasma-mass spectrometry (ICP-MS) and mass spectrometry (GC-MS) to simultaneously identify and quantify organic forms of selenium. These methods are also quite accurate with a very good LOD, a wide range of linearity, and a high selectivity. Furthermore, these methods are fast and detect more selenium species, especially nonvolatile selenium compounds after derivatization. Indeed, in biological and food samples, selenium is mostly present in the form of nonvolatile compounds, such as organic and inorganic species, small molecules, seleno-amino acids, and polypeptides. These HPLC-ICP-MS/GC-MS methods are utilized in the assessment of total selenium contents after performing the required extraction protocols, which itself yields very complex hydrolysates [74, 75].

A study reported comparing two hydrolysis methods for the simultaneous quantification of Met and SeMet, employing either Protease XIV or methanesulfonic acid (MA) in a reflux system or a microwave oven, followed by diethyl ethoxymethylene malonate derivatization. In this method, hydrolysis is then followed by analysis with multiple reactions monitoring HPLC in tandem mass spectrometry (MRM-HPLC-MS/MS) [76]. The HPLC system is equipped with a diode array detector and a high-resolution mass spectrometer with an electrospray ionization (ESI) source. Analyte separations are carried out on a normal phase column at 30 °C. Nitrogen is added as the collision gas for selected fragmentation of molecular ions. Both approaches can determine selenium/sulfur substitutions accurately in Met. When compared to common methods, such as (GC-MS) or (ICP-MS), the second approach exhibits accurate simultaneous quantification of Met and SeMet with a low LOD, a low LOQ and wide linearity range. This new method is validated using certified reference material conjoined with the GC-MS reference method [76]. These methods, which are based on hydrolysis represent a promising alternative for the elucidation of selenium substitution reactions. This is indeed the case when the aim is to achieve high precision, high reproducibility, and high accuracy for samples containing low amounts of Met and SeMet in the range of 0.1-25 μg/mL, for example. The main drawbacks here are lower recovery and sensitivity in comparison to spectrophotometric methods of quantification.

Undoubtedly, the determination of selenium in biological samples is challenging, especially because the element is usually present in trace amounts. Considerable efforts are therefore being dedicated to develop sensitive and applicable methods, which contribute to the assessment of selenium at concentrations in the human body or even in unveiling new substances based on this element. For example, methods used for the analysis of serum selenoalbumin (SeAlb), selenoprotein P (SelP), and glutathione (GPx) depend on the combination of affinity HPLC (AFHPLC) for protein separation with on-line selenium detection by ICP-quadrupole MS (ICP-QMS) or collision/reaction cell ICP-QMS (CRC ICP-QMS) [75, 77, 78].

A systematic approach to the accurate quantification of selenium in serum selenoalbumin (SeAlb) by HPLC-ICP-MS has been proposed and two different methods have been designated for this task [75]. The first method is based on the enzymatic hydrolysis of the entire serum with a mixture of lipase proteases followed by the determination of SeMet extracted from SeAlb, employing species-specific isotope dilution (SSID) ($^{80}Se/^{76}Se$ ratio). SSID helps to reduce the potential interference of species transformation, which might occur during any step of the analytical procedure and affect, in turn, the accuracy of results. Subsequently, the sample is separated by reverse-phase RP-HPLC coupled to CRC ICP-QMS. The advantage of RP-HPLC is an excellent separation of SeMet without the need for an additional purification step, requiring neither size exclusion HPLC (SE-HPLC) nor centrifugation. The second method has been designed to assess the yield of enzymatic hydrolysis. In this method, SeAlb is determined as an intact protein by affinity HPLC coupled to CRC ICP-QMS. The same method also has been considered in the determination, within the same chromatographic run, of glutathione peroxidase (GPx) and selenoprotein P (SelP), the two other major selenoproteins present in human serum. Separation of intact SeAlb and selenoproteins was performed in undiluted human serum utilising a double-column AF-HPLC consisting of Heparin-Sepharose (HEP) and Blue-Sepharose (BLUE) column system, taking advantage of the selectivity of proteins towards HEB and BLUE stationary phases. Thereafter, the chromatogram presented peaks for GPx, SelP, and SeAlb gained by analysis of undiluted human serum using AF-HPLC coupled to CRC ICP-QMS [75].

Other methods have also been employed for the detection of different selenium species, with different analytical techniques, such as atomically imprinted polymer (AIP) and hydride generation atomic absorption spectrometry (HG-AAS). Atomically imprinted polymer (AIP) has been developed for the on-line preconcentration/determination of selenium and has been coupled to a spectrometric flow injection analysis hydride generation flame atomic absorption spectrometry (FIA-HG-FAAS) system. This marks a novel approach where the AIP shows significant sensitivity with a 53 ng/L LOD and a high preconcentration factor. Furthermore, this impressive low LOD is considered the best in literature, with the potential of even further improvement by increasing the preconcentration period [79]. These outcomes constitute a reliable alternative method for determining the concentration of selenium in different kinds of samples. The validation of the method has been investigated concurrently during the analysis of Brazil nuts, apricot, milk powder, rice flour, and white beans. Results concord with addition and recovery tests employing standard selenium concentrations. Recovery

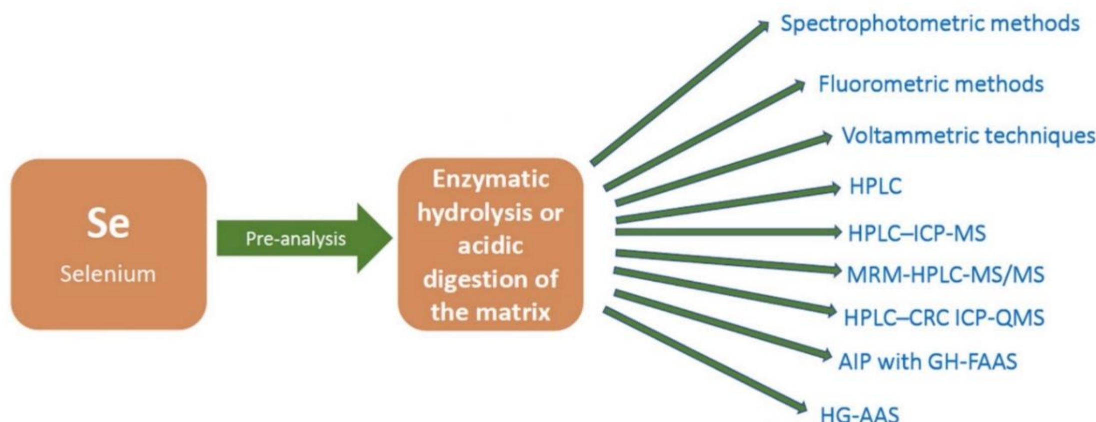


Fig. (2). A summary of common analytical methods used for selenium speciation and quantification (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

Table 2. Comparison of quantification methods for selenium and their respective limits of detection (LOD) and quantification (LO-Q).

Method of Quantification	Species Detected	LOD	LOQ
HPLC (based on the König reaction)	Seleno-cyanate ($SeCN^-$)	73.5 fmol/mL	245.1 fmol/mL
MRM-HPLC-MS/MS without derivatization	Seleno-me-thionine	0.03 µg/mL	0.10 µg/mL
MRM-HPLC-MS/MS with derivatization	Seleno-me-thionine	0.01 µg/mL	0.03 µg/mL
HPLC-CRC ICP-QMS	Seleno-albumin	0.14 - 0.20 ng/g (Se to the mass of serum)	-
AIP with GH-FAAS	Precon-centrated Se	53 ng/L	177 ng/L
HG-AAS	Se(VI)	0.78 mg/L	2.35 mg/L

percentages are in the range of 98-109% and the accuracy of the method has 95% confidence.

Moreover, this new method exhibits good precision with relative standard deviations of less than 10%. It should be noted that when some interferents are present, mainly due to Cu^{2+} and Cr^{3+}, an additional standard calibration is recommended where the impact of the interfering ions is accounted for by binary mixtures containing 20 µg/L of selenium and the concomitant [79]. A similar method employing hydride-generation atomic absorption spectrometry (HG-AAS) has been investigated to determine selenium content in chicken eggs and chicken meat [80]. In these products, the method shows an LOD of 0.78 mg/L and an LOQ of 2.35 mg/L. Moreover, the accuracy and precision of the method are in an acceptable range with a relative standard deviation of less than 4%, while the relative error is below 10% for a sample of 500 mL and selenium content of 10 mg/L [80].

Fig. (**2**) summarizes the aforementioned separation and analytical methods and Table **2** compares these methods according to their LOD and LOQ.

CONCLUSION

The importance of selenium in biology has emphasized the need to develop and investigate cutting-edge analytical methods for this chalcogen element. Indeed, many valid approaches exist, and the ones highlighted in this review designate a fair share of the most reliable ones. The review at hand is comprehensive in the sense that it presents different approaches varying in protocol, accuracy, sensitivity, reproducibility and economic cost. It should also be noted that dealing with biological or food-based samples requires careful preanalytical steps and that certified reference materials are scarce.

Analytical methods and techniques mentioned in this review, therefore, should be employed at the right place for the right sample. While neutron activation analysis and HPLC-ICP-MS methods have provided the best outcomes, they are often rather tedious, expensive, and require skilled personnel. Spectrophotometric and fluorometric methods are widely employed, yet they suffer from low sensitivity. The need for lower detection limits and the low range of detectable molecules are limiting points of these techniques. The straightforward and affordable voltammetric techniques suffer a degree of inaccuracy and, in some instances, are in-

applicable to detect selenium at low concentrations in biological samples due to matrix interferences and overlapping of signals. Electrothermal atomic absorption spectrometry techniques are commonly applied due to their promising sensitivity, selectivity, low limit of detection, and the small volume required to conduct experiments. Unfortunately, the spectral interference associated with the latter techniques might pose an issue for the overall determination. Therefore, many methods rely on combinations of several techniques to achieve better results, such as HG-AAS, hydride-generation atomic fluorescence spectrometry, or hydride-generation ICP-optical emission spectrometry, where the usual interferences are minimized significantly.

Despite the enormous efforts and research initiatives in the field of selenium speciation, there is yet a lot to unveil and optimize, likewise, to innovate methods in which the molecules are less affected and the outcomes are more accurate.

CONSENT FOR PUBLICATION

Not applicable.

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CONFLICT OF INTEREST

Pr Dr. Claus Jacob and Dr Caroline Gaucher are Editorial Board Members for the Journal Current Nutraceuticals.

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1.8 New selenohydantoins inspired by the heart of tuna, to treat heart diseases

Naturally occurring organoselenium molecules are important sources of nutritional selenium and are important examples and analogues for many synthetic organoselenium molecules inspired by nature, such as the marine-originated selenoneine. While on one side, selenoneine has generated interest for the potential health benefits that have been already discussed, on the other side, it raises ethical and sustainability concerns. Indeed, it is primarily obtained from marine organisms where it is present at low concentration posing problems of overharvesting or harming marine ecosystems in the quest to obtain this molecule. Unfortunately, there are also several challenges associated with its chemical synthesis such as a complex chemical structure, and a lengthy synthesis pathway. Indeed, selenoneine contains a selenium atom that is bound uniquely within a heterocyclic ring, which renders this precise arrangement challenging to achieve in a laboratory setting and may require specialized synthetic techniques. Moreover, selenium molecules are known for their reactivity and sensitivity to oxidation. Selenoneine, like other selenium-containing molecules, can be prone to chemical degradation if not handled and stored properly. Maintaining its stability during synthesis and purification processes can be difficult. Due to these factors and relatively recent discoveries, only two established methods are available for selenoneine synthesis in the literature [128–130]. However, those protocols are time consuming, complicated and produce low yields. In contrast, carrying out the synthesis on really large scales is expensive and may not be feasible, hence, making it less accessible for research and potential commercial applications. Moreover, analysing and verifying molecules identity and purity can be challenging due to its complex structure, instability, and potential impurities that may arise during synthesis. Thus, overcoming these challenges requires interdisciplinary efforts from chemists, biologists, and environmental science researchers and may involve developing innovative synthetic methods to address ethical and regulatory considerations.

Therefore, simplifying the chemical structure by focusing on simpler organoselenium molecules allows researchers to work with more manageable and stable molecules, reducing the difficulties associated with its synthesis and handling. Simple organoselenium molecules are generally more accessible and available in larger quantities than complex molecules like selenoneine. This accessibility facilitates research and experimentation, making it easier to investigate their properties and potential applications. As mentioned earlier, selenoneine is a complex selenium-containing molecule with a unique structure. As a result, there have been many efforts in synthesizing selenium-containing molecules that have antioxidant and redox modulation activity, such as selenohistidine and selenohydantoin derivatives, which both share a basic seleno-imidazole ring structure with selenoneine (Figure 9) [131–133]. These derivatives have a promising range of pharmacological

applications, such as anti-inflammatory, anticancer, and antiplatelet agents, and also provide a relatively more straightforward synthesis and good yields [131,132,134].

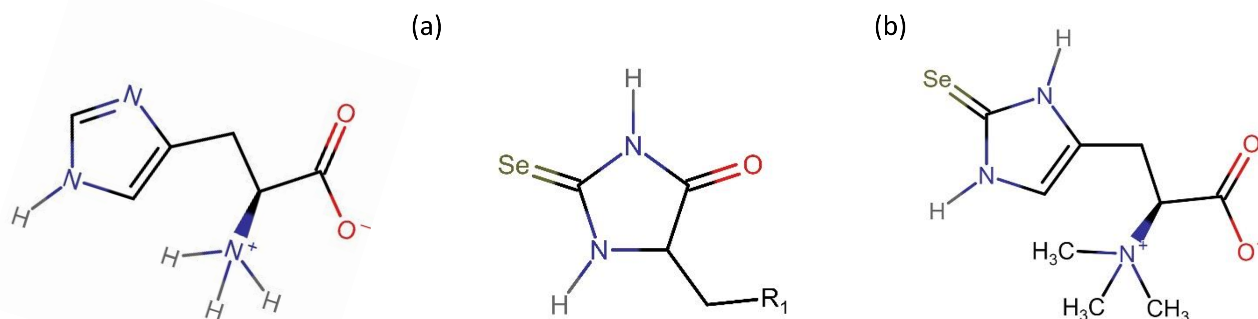

Figure 9: *Chemical structures of (a) Histidine ©, (b) Selenohydantoin, and (c) Selenoneine ©.*

Indeed, selenium-containing molecules, such as selenohydantoins and ebselen may help improve endothelial function and increase •NO bioavailability [118,135–137]. Studies suggest that endothelial dysfunction, a hallmark of many CVDs, could be a direct result of vascular oxidative stress and chronic inflammation, and that molecules mitigating inflammation may have protective effects on the cardiovascular system. Interestingly, organoselenium molecules such as GPx mimics induce a significant reduction of inflammation and vascular oxidative stress. In addition, they prevent down-regulation of eNOS leading to sustained eNOS levels and normal vascular function, thus restoring vascular endothelial function [136]. Organoselenium molecules can also release •NO from its carriers, thereby increasing the •NO bioavailability for vasodilation and protein *S*-nitrosation [118,137].
Consequently, the synthesis and evaluation of various selenium-containing molecules, for instance selenohydantoins, is promising. These molecules have already been explored in the literature, demonstrating two-fold higher anticancer activity *in vitro* compared to thiohydantoins. Interestingly, the incorporation of selenium into the hydantoin scaffold shows great potential for producing molecules with enhanced medicinal properties, thus making selenohydantoins a promising avenue for drug development research. Additionally, these derivatives have shown promise in terms of antioxidant and GPx-like activities, further highlighting their potential for therapeutic applications.

Selenohydantoins can be synthesized through various chemical routes, allowing the exploration of different synthetic strategies and in order to optimize their production. This versatility makes them attractive targets for synthetic chemists. The structure of selenohydantoins can be modified by introducing various substituents or functional groups, potentially tailoring their properties for specific applications.

Selenohydantoins and selenoneine are both with distinct structures and potential roles in human health. Furthermore, their potential importance in cardiovascular diseases is an intriguing area of research. As already stated, the potential activities in CVDs stem from the antioxidant properties and the redox modulating ability of selenohydantoins and the potential cardioprotective effects of these molecules are promising.

While there are evidences to suggest that selenium supplementation may reduce the risk of certain cardiovascular events, more studies are needed to determine the specific contributions of the redox modulating and anti-inflammatory properties of selenohydantoins that could potentially be employed in this context

1.9 Ph.D. objectives

The primary objectives of my doctoral research project were centred around advancing novel strategies and solutions to reduce oxidative stress and to enhance $^{\bullet}$NO bioavailability in the context of cardiovascular health. Specifically, my work focuses on the synthesis and the evaluation of selenohydantoins, a class of selenium-containing molecules inspired by selenoneine. From a chemical perspective, these selenohydantoins offered additional advantages compared to other known selenium molecules, such as ebselen, including increased solubility in an aqueous environment and simplified synthesis compared to selenoneine itself.

The investigation encompassed an assessment of the selenohydantoins' potential as antioxidants with redox-modulating properties and capacity for GPx-like activity. Of particular interest was their ability to facilitate the release of $^{\bullet}$NO from GSNO through redox-modulating thiol exchange reactions and the ability to induce protein *S*-nitrosation [118].

To achieve these research goals, we developed and elucidated straightforward synthesis protocols for a new series of selenohydantoins. Characterization of this series involved various physicochemical analytical techniques, including the determination of synthetic yields, nuclear magnetic resonance (NMR) spectroscopy, high-resolution mass spectrometry (HRMS), melting point measurements, ultraviolet-visible (UV-Vis) spectral analysis (λ_{max}), and calculation of the logarithm of the partition coefficient (cLog*P*).

To assess the efficacy of these selenohydantoins, comprehensive evaluations were conducted, comparing their antioxidant and GPx-like activities as well as redox-modulating capabilities to well-established controls, such as ebselen, GSH, ascorbic acid, and selenocystamine. Additionally, theoretical calculations employing density functional theory (DFT) were performed to gain insights into the possible existence of multiple tautomers of selenohydantoins in solution.

Based on the promising outcomes of the *in tubo* evaluations, selenohydantoins demonstrating the most favourable activities were selected for further *in vitro* and *ex vivo* assessments. These subsequent investigations aimed to evaluate their impact on protein *S*-nitrosation and antioxidant processes *in vitro* by assessing GPx activity and intracellular GSH quantification, utilizing HuAoSMCs. Furthermore, their capacity to mobilize •NO reserves and to influence vasoreactivity was explored *ex vivo* using rat aortic rings

2 Chapter II: Synthesis and evaluation of new selenohydantoins

2.1 Chemical Synthesis

Given the significance of selenoneine, our research endeavours have cantered on its synthesis and isolation, as well as the development of a series of molecules inspired by this intriguing selenium-containing molecule. Although the chemical synthesis of selenoneine has been previously documented by Li Wei *et al*. [128] as discussed in Chapter I, the synthesis protocol is notably intricate. Unfortunately, in our hands it was unsuccessful in yielding the desired molecule primarily due to two main challenges: i) The initial material (2,5-dibenzoyl amido-4-ketone methyl valerate) is not commercially available and its synthesis and isolation proved to be a complicated task. ii) Reactions were conducted on a kilogram scale, a magnitude not practical within the confines of a research laboratory, and involving numerous steps. Alternatively, reducing the synthesis scale to milligrams or even in grams has caused the protocol to fail and made it unfeasible to pursue the synthesis due to very low yields. Moreover, the costs associated would be prohibitively high for the completion of these reaction steps. To avoid the previous issues, we adjusted the synthesis pathway, as illustrated in Figure 10, by commencing directly with histidine. Regrettably, solubility issues arose, significantly impacting reaction yields and thus hindering the progression and success of this modified, alternative pathway.

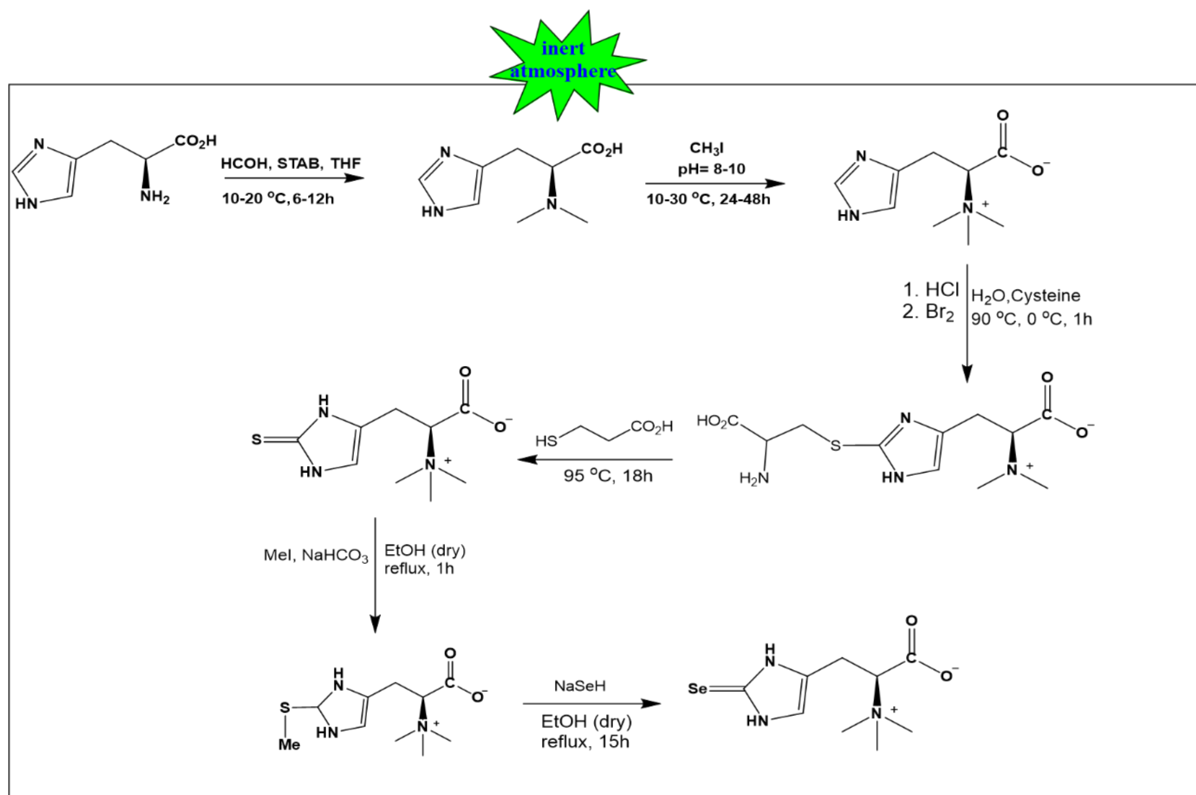

Figure 10: *A proposed protocol for selenoneine synthesis starting from histidine.*

An alternative synthesis protocol was developed by D. Lim *et al*.[138], the synthesis of selenoneine started with l-histidine methyl ester with and produced selenoneine, which was isolated as diselenide in 2% yield (120 mg) over 11 steps. However, this approach proved to be unpractical due to the protocol's complexity, involving many steps, and resulting in very low yields of the target molecule. Subsequently, we approached a different route by starting with ergothioneine Figure 11, a naturally occurring antioxidant found in mushroom varieties. Ergothioneine serves as a selenoneine analogue, with sulphur in its chemical structure instead of selenium.

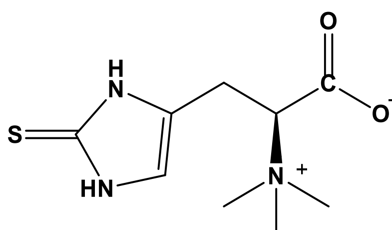

Figure 11: *Chemical structure of Ergothioneine*

Our synthesis journey began with a two-step protocol, Figure 12, commencing with *S*-alkylation, followed by a selenation step applied to ergothioneine, in order to obtain selenoneine. At the same time, we were approached by Tetrahedron Company (Paris, France), expressing their interest in the potential synthesis efforts and offering to provide ergothioneine samples.

Further, the first step yielded *S*-methyl ergothioneine, which was confirmed by ^{1}H, ^{13}C NMR, and HPLC-MS with very minor impurities. However, when analysing the selenated molecule (potential selenoneine) *via* NMR, we encountered numerous impurities. To address this, we continued with further purification through column chromatography (using silica, 10:2:1 MeOH: H_2O:30 % aqueous NH_4OH). Subsequent ^{1}H and ^{13}C NMR and ^{13}C NMR analysis showed the possible presence of the selenoneine diselenide form with fewer amounts of impurities. However, ^{77}Se NMR failed to prove the presence of selenium.

To improve purity, we explored an alternate purification method. This involved the addition of a small quantity of water to the reaction flask, followed by evaporation. The resulting product was then placed in an Erlenmeyer flask with ethanol, heated to boiling, and filtered directly while still hot. This procedure was repeated twice, followed by either the product's recrystallization or the solvent's evaporation. Nevertheless, our approach faced several challenges, including low reaction concentration due to the high cost of the starting material (ergothioneine), which in turn resulted in a very low yield. This in turn limited the quantity available for NMR analysis and also hindered our ability to run purification methods to obtain an ultra-pure molecule.

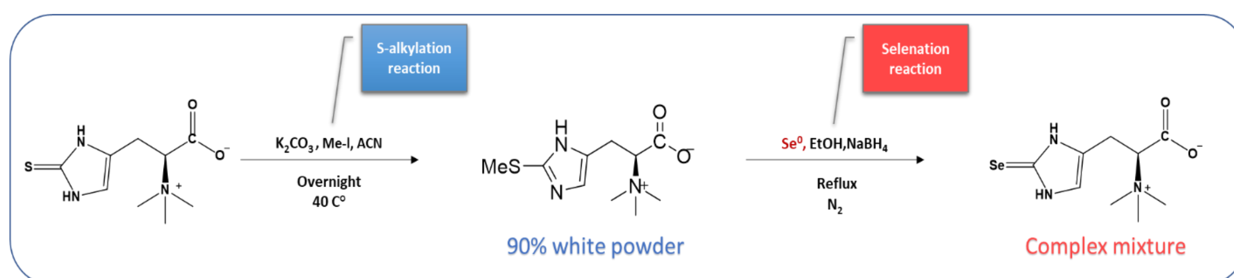

Figure 12: *A two-step synthesis protocol of selenoneine starting with Ergothioneine.*

Considering the difficulties of synthesizing selenoneine and the importance of this molecule within the project, we were driven to design molecules with more straightforward chemical synthesis pathways. We aimed to target a group of derivatives that would be easier to achieve while ensuring enhanced stability and solubility. Subsequently, we successfully synthesized a series of new selenohydantoins,

which indeed share a basic seleno-imidazole ring structure with selenoneine, moreover, the presence of a carbonyl group on the ring might further contribute to better stability and reactivity of the molecule. In addition, the simplicity to synthesize different derivatives, and the fact that senelohydantoins derivatives have been already investigated for their potential therapeutic uses has let us to focus on this group of molecules [139].

These new molecules underwent rigorous characterization through physicochemical methods and were subjected to evaluation for their antioxidant and GPx-like activity, the results of which has been published in *Bioorganic Medicinal Chemistry* journal in 2023.

Article IV: **Selenoneine-inspired selenohydantoins with glutathione peroxidase-like activity**

Rama Alhasan, Guilherme M. Martins, Pedro P. de Castro, Rahman Shah Zaib Saleem, Ali Zaiter, Isabelle Fries-Raeth, Alexandra Kleinclauss, Caroline Perrin-Sarrado, Patrick Chaimbault, Eufrˆanio N. da Silva Júnior, Caroline Gaucher, Claus Jacob

Bioorganic & Medicinal Chemistry, 2023, 94, 117479.

Chronic diseases such as cystic fibrosis, inflammatory bowel diseases, rheumatoid arthritis, and cardiovascular illness have been linked to a decrease in selenium levels and an increase in oxidative stress. Selenium is an essential trace element that exhibits antioxidant properties, with selenocysteine enzymes like glutathione peroxidase being particularly effective at reducing peroxides. In this study, a series of synthetic organoselenium molecules were synthesized and evaluated for their potential antioxidant activities. The new selenohydantoin molecules were inspired by selenoneine and synthesized using straightforward methods. Their antioxidant potential was evaluated and proven using classical radical scavenging and metal-reducing methods. The selenohydantoin derivatives exhibited glutathione peroxidase-like activity, reducing hydroperoxides. Theoretical calculations using Density Functional Theory (DFT) revealed the selenone isomer to be the only one occurring in solution, with selenolate as a possible tautomeric form in the presence of a basic species. Cytocompatibility assays indicated that the selenohydantoin derivatives were non-toxic to primary human aortic smooth muscle cells, paving the way for further biological evaluations of their antioxidant activity. The results suggest that selenohydantoin derivatives with trifluoro-methyl (-CF_3) and chlorine (-Cl) substituents have significant activities and could be potential candidates for further biological trials. These molecules may contribute to the development of effective therapies for chronic diseases such cardiovascular diseases.

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Supplementary data file is available here: https://ars-els-cdn-com.bases-doc.univ-lorraine.fr/content/image/1-s2.0-S0968089623003279-mmc1.doc

Selenoneine-inspired selenohydantoins with glutathione peroxidase-like activity

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Abstract: Chronic diseases such as cystic fibrosis, inflammatory bowel diseases, rheumatoid arthritis, and cardiovascular illness have been linked to a decrease in selenium levels and an increase in oxidative stress. Selenium is an essential trace element that exhibits antioxidant properties, with selenocysteine enzymes like glutathione peroxidase being particularly effective at reducing peroxides. In this study, a series of synthetic organoselenium compounds were synthesized and evaluated for their potential antioxidant activities. The new selenohydantoin molecules were inspired by selenoneine and synthesized using straightforward methods. Their antioxidant potential was evaluated and proven using classical radical scavenging and metal-reducing methods. The selenohydantoin derivatives exhibited glutathione peroxidase-like activity, reducing hydroperoxides. Theoretical calculations using Density Functional Theory (DFT) revealed the selenone isomer to be the only one occurring in solution, with selenolate as a possible tautomeric form in the presence of a basic species. Cytocompatibility assays indicated that the selenohydantoin derivatives were non-toxic to primary human aortic smooth muscle cells, paving the way for further biological evaluations of their antioxidant activity. The results suggest that selenohydantoin derivatives with trifluoro-methyl ($-CF_3$) and chlorine (-Cl) substituents have significant activities and could be potential candidates for further biological trials. These compounds may contribute to the development of effective therapies for chronic diseases such cardiovascular diseases.

Keywords: Selenium, Antioxidant, Redox, Glutathione Peroxidase, Selenohydantoins, Human Aortic Smooth Muscle.

1. Introduction

Selenium (Se) was discovered as an essential element in 1818 and later identified in the 1960s by Schwarz and Foltz as being essential for protecting rat livers from necrosis.[1] A few years later, it was also confirmed as an essential nutrient for human health.[2] Although humans obtain most of their dietary Se from sources such as bread, cereal, meat, seafood, and poultry, the physiological levels of Se are directly linked to its availability in soil, which is influenced by its geographical distribution.[3,4] Indeed, Se deficiency affects 3 billion people worldwide and is responsible for cancers, liver diseases, and inflammation, and can also influence the production of thyroid hormones. The Institute of Medicine (IOM) of the National Academies (United States) recommends a dietary allowance (RDA) of 55 μg of Se per day for adults over 19 years of age. Pregnant and lactating women require about 60 and 70 μg per day, respectively. In 2014, the European Authority for Food Safety (EFSA) set the RDA for Se for adults at 70 μg per day, while the usual intakes vary between 31 and 65.6 μg per day in Western Europe. It is also important to note that excessive consumption of Se can lead to complications such as fever, vomiting, nausea, diarrhea, hair loss, and more severe symptoms, including liver and kidney problems.[5] Therefore, this fine line between the good and the bad side of Se needs to be monitored closely.

Environmentally speaking, Se is found in different forms, with the most abundant ones being selenite (SeO_3^{2-}, Se(IV)), selenate (SeO_4^{2-}, Se(VI)), selenides (Se^{2-}), and elemental selenium (Se^0). Among these forms, Se(IV) is generally considered more toxic than Se(VI).[6,7] Organoselenium compounds are physiological forms of Se in living organisms such as selenoproteins in mammals or selenoneine, the major form of organic Se found in tuna.[8] Moreover, 25 selenoproteins have been identified so far within the human body including the glutathione peroxidase (GPx) and the thioredoxin reductase (TrxR) families with antioxidant and anti-inflammatory activities.[9,10] Glutathione peroxidases form a family of multiple seleno-isozymes which catalyze the reduction of hydrogen peroxide (H_2O_2) or organic hydroperoxides to water or corresponding alcohols, respectively, using reduced glutathione (GSH) as an electron donor

(H_2O_2 + 2 GSH → GSSG + 2 H_2O).[11–16] Se therefore plays a critical role in mammalian seleno-enzymes as it confers the antioxidant catalytic activity in GPx.[17,18] The selenocysteine residue, with a selenol group (–SeH) having a p*Ka* of 5.5 at physiological pH, exhibits superior redox properties compared to the cysteine residue's thiol group. This is because the selenol group is more nucleophilic, making it a better nucleophile for attacking electrophilic substrates in enzymatic reactions. Additionally, the selenol group's lower p*Ka* compared to the thiol group allows it to exist in its deprotonated form at physiological pH, making it more reactive.[19] Studies have shown that replacing selenocysteine with cysteine in selenoproteins results in reduced enzymatic activity, supporting the idea that a deprotonated chalcogen atom is essential for proper function.[20,21]

Organoselenium compounds that mimic the activity of GPx have gained tremendous interest as antioxidants, regulating free radical reactions in the organism.[22–24] Ebselen is the flagship organoselenium compound that mimics GPx activity.[25] Unfortunately, ebselen has poor aqueous solubility (log*P* around 3.7) and therefore requires special delivery approaches to be evaluated in biological systems. Aqueous solubility is an important quality to consider when synthesizing selenocompounds. For example, notable approaches have been developed in synthesizing selenosugars that have high solubility.[26] Other potent organoselenium molecules have also been reported, such as ethaselen, a mammalian thioredoxin reductase 1 inhibitor with antitumor activity; 4,4-dimethyl-benziso-2H-selenazine (ALT 2074), which exhibits anti-inflammatory activity; and diseleno-*bis*-Benzamides, which have antiviral activities.[27–30] Furthermore, in 2010, selenoneine, an unusual derivative of seleno-histidine, was isolated from the blood and tissues of tuna fish. Selenoneine contains a selenium atom on the imidazole ring and is also the selenium analog of ergothioneine. This natural organoselenium compound has significant antioxidant activity and may have a wide range of therapeutic potentials. However, investigations into the properties of this novel molecule are limited due to its hydrophilic nature, with a log*P* of -9.01, which complicates its isolation from biological samples, whereas its cumbersome multistep chemical synthesis generally resultsvery low yields.[8]

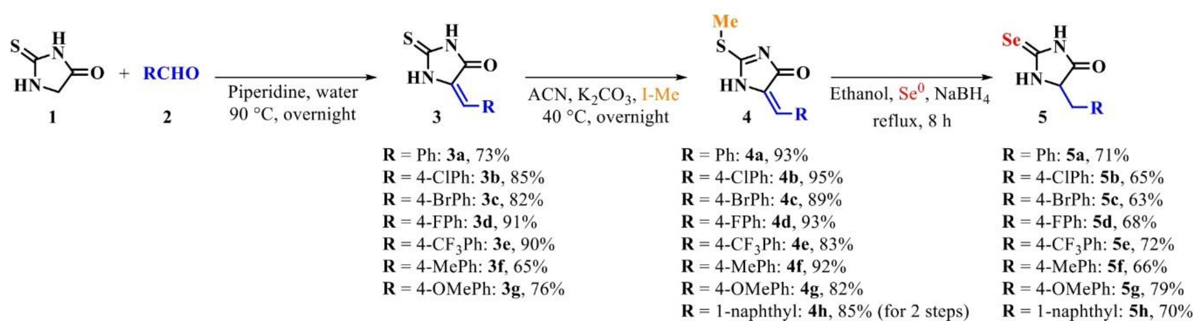
Recently, there have been several efforts in synthesizing Se-containing molecules that possess antioxidant and redox modulation activity, such as selenohistidine and selenohydantoin

derivatives, which both share a basic seleno-imidazole ring structure with selenoneine.[8,31–33] These derivatives have an interesting range of pharmacological applications, *i.e.* as potential anti-inflammatory, anticancer, and antiplatelet agents, and also rely on a relatively more straightforward synthesis and good yields, thus representing prominent target molecules.[31,32,34] Indeed, Ivanenkov *et al.* have previously synthesized and studied a series of selenohydantoin derivatives, which exhibit two-fold higher anticancer activity *in vitro* than thiohydantoins. Furthermore, they have also found promising antioxidant and GPx-like activities.[34]

In this study, we initially focused on synthesizing a series of molecules based on the selenoneine structure. Moreover, the goal was to design a redox modulator molecule having good solubility in biological systems. Thereafter, we investigated the antioxidant and GPx-like activities and also cytocompatibility with human aortic smooth muscle cells. The series of selenohydantoin derivatives obtained with a selenium atom on the imidazole ring had both, good yields and fairly better solubility than ebselen, and also showed promising redox modulating activities, especially in the case of molecules with trifluoromethyl group (-CF_3) and chlorine (-Cl) substituents.

2. Results and Discussion

The synthesis of 5-aryl-2-selenoxoimidazolidin-4-one derivatives **5a**–**5h** was conducted in three steps as shown in Scheme 1.



Scheme 1. General synthetic route for the synthesis of selenohydantoin derivatives.

Initially, the condensation reaction between 2-thiohydantoin **1** and appropriate aldehydes **2** was performed using piperidine as base and water as the solvent, at 90 °C overnight. The prod-

uct was precipitated from the reaction mixture and was purified by repeated washing with diethyl ether to afford **3a**–**3h** in 65–91% yield. Then, compounds **3a-3h** were treated with iodomethane under basic K_2CO_3 condition in acetonitrile as solvent at 40 °C to provide *S*-methyl derivatives **4a-h** in yields of 82–95%. Compounds were purified by chromatographic column. 5-Aryl-2-selenoxoimidazolidin-4-one derivatives were obtained by reacting (*Z*)-5-arylidene-2-(methylthio)-3,5-dihydro-4*H*-imidazol-4-ones **4a-h** with nucleophilic selenium species (NaSeH).[35,36] The anion is added to the carbon atom of the isothiourea, followed by the elimination of methanethiolate as a leaving group.[37] The reaction mixture was refluxed for 8 h, and the selenylation reaction and the reduction of sp^2 carbon were carried out *in situ*, delivering **5a-h** in yields of 63-79%. Products were purified by column chromatography. The NMR spectra and the exact high-resolution masses are provided in the supplementary data, S2 to S24. Physiochemical parameters of each selenohydantoin derivative such as the melting point, the cLog*P*, and the maximum peak of absorbance (λmax Abs) were also specified (Table 1).

Table 1

Physiochemical properties of selenohydantoin derivatives, including, calculated Log*P* (cLog*P*) and the maximum peak of absorbance λ_{max} Abs

Compounds	cLog*P*	λ_{max} Abs (nm)
5a	0.293	288
5b	1.006	288
5c	1.156	286
5d	0.436	288
5e	1.175	289
5f	0.791	264
5g	0.293	287
5h	1.467	291
Ebselen	3.700	327 [38]
Selenoneine	-9.012	260 [39]

The selenohydantoin derivatives showed similar maximum absorption values (λmax). However, their cLog*P* values, calculated in order to determine their hydrophilicity, varied considerably. A lower cLog*P* indicates better aqueous solubility. Interestingly, the selenohydantoin derivatives synthesized

as part of this study have lower cLog*P* values than ebselen, which suggests that they may have improved aqueous solubility. On the other hand, there are various rules to predict the good bioavailability, and one of the most well-known is the Lipinski rule. This rule depends on to the molecular properties which are important for a drug's pharmacokinetics in the human body such as absorption, distribution, metabolism, and excretion (ADME).[40] Selenohydantoin derivatives are still less water soluble than selenoneine, which is important for handling and bioavailability. Selenohydantoin derivatives therefore represent a compromise between ebselen and selenoneine as on the contrary of ebselen, they are soluble in biological media and they might be more stable than selenoneine in those media. The newly synthesized selenohydantoin derivatives were subjected to further evaluation to determine their antioxidant activity, including their ability to scavenge radicals and reduce ferric ions. Since ebselen did not exhibit any capacity to reduce DPPH$^{\bullet}$, the selenohydantoin derivatives were compared to two well-known positive controls, ascorbic acid and reduced GSH. These compounds are highly valued for their strong antioxidant properties, which arise from their ability to donate electrons that neutralize and stabilize free radicals.[41–43] The corresponding EC_{50} values were determined, plus the stoichiometric coefficients of the reaction (Table 2).

Table 2

Akinetic parameters calculated from the DPPH assay. Effective concentration 50 (EC_{50}) of selenohydantoin derivatives and controls regarding DPPH$^{\bullet}$ reduction and related stoichiometry coefficient between a selenohydantoin derivative or control molecules *versus* DPPH$^{\bullet}$. Results are presented as means ± sem, n = 3 and are compared using One-way ANOVA, and Bonferroni post-test, * $p < 0.05$ *versus* ascorbic acid, # p < 0.05 *versus* GSH.

Compounds	EC_{50} (µM)	Stoichiometric coefficient
5a	**37 ± 3** *,#	1
5b	**44 ± 2** *	1.2
5c	60 ± 3 *,#	1.7
5d	**39 ± 1** *,#	1.1
5e	**35 ± 1** *,#	1
5f	89± 2 *,#	2.5
5g	**38 ± 3** *,#	1.1
5h	44 ± 1 *	1.3

Ascorbic Acid	17.8 ± 0.1 #	0.5
GSH	50 ± 1 *	1.4
Ebselen	No activity	-

Each selenohydantoin derivative exhibited lower DPPH$^{•}$ scavenging activity than ascorbic acid. Nonetheless, **5a**, **5b**, **5d**, **5e** and **5g** were statistically more efficient to reduce DPPH$^{•}$ than GSH with a lower stoichiometric coefficient (close to 1) than GSH. This is likely due to the selenium atom in the selenohydantoin derivatives being more reactive than the sulfur atom in GSH, as selenium has a lower *pKa* and undergoes faster electrophilic and nucleophilic substitutions. Only **5c** and **5f** are less effective than GSH with around 2 molecules needed to reduce one DPPH. Overall, these results suggest that most of the synthesized selenohydantoin derivatives may have potential as new antioxidants.

The antioxidant activity of many compounds can be attributed to both SET and HAT, especially in the presence of the selenone-selenol tautomerism, the selenolate, and the NH-bond. Moreover, the presence of electron-withdrawing groups such as -CF_3 in **5e,** OMe in **5g,** or phenyl in **5a** can contribute also to the activity by affecting the bond dissociation energy, promoting the HAT mechanism.[44] On the other hand, the presence of a halogen group such as -Cl, -Br, and -F in **5b**, **5c,** and **5d**, respectively, may contribute to the increase of antioxidant activity related to SET. For instance, the derivative with -CF_3 substitution exhibits an EC_{50} value of 35 ± 1 µM. As a result, the kinetics of the reaction were further investigated for selenohydantoin derivatives showing an EC_{50} significantly lower than GSH. Figure 1 shows the fitting of reaction curves to the equation of the non-linear *pseudo*-first-order reaction rate.

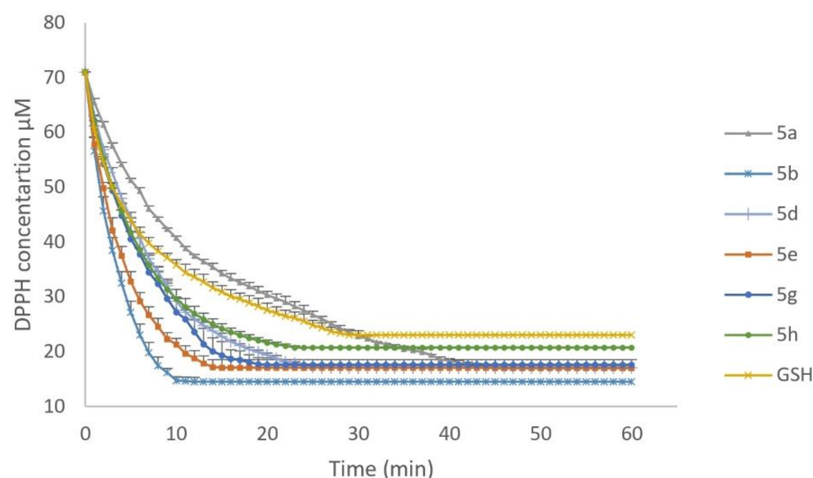


Figure 1. Kinetic curves of DPPH reduction by fixed concentration (EC_{50}) of selenohydantoin derivatives and GSH and their fitting to the equation of non-linear pseudo-first-order reaction rate ($y = a\ e^{-bx}$).

In this study, molecules at a fixed concentration (EC_{50}) were left to react for 60 min and calculations were made based on the time needed to reach completion of the reaction. The efficiency of the antioxidants was determined based on the kinetics of the reaction, with faster reactions indicating more efficient antioxidants. The kinetic parameters were determined based on the curve fitting shown in Figure 1 and detailed in Table 3.

Table 3

Kinetic parameters such as time to reach reaction completion (TEC_{50}), antiradical efficiency (AE), and rate constants k_1 and k_2 calculated from DPPH reduction by fixed concentration (EC_{50}) of selenohydantoin derivatives and GSH. Results are presented as means ± sem, n = 3, and are compared using One-way ANOVA, and Bonferroni post-test, * $p < 0.05$ *versus* GSH.

Compounds	TEC_{50} (min)	AE x 10^{-3} ($min^{-1}.\mu M^{-1}$)	k_1 ($\mu M^{-1}\ min^{-1}$)	k_2 ($M^{-1}\ s^{-1}$)
5a	42 ± 1 *	0.63 ± 0.01	0.46 ± 0.01	0.46 ± 0.01
5b	11 ± 1 *	2.1 ± 0.1 *	1.91 ± 0.03 *	2.28 ± 0.04 *
5d	24 ± 1 *	1.05 ± 0.04 *	0.81 ± 0.02 *	0.89 ± 0.02 *
5e	14 ± 1 *	2.1 ± 0.1 *	1.6 ± 0.1 *	1.6 ± 0.1 *
5g	18 ± 1 *	1.4 ± 0.1 *	1.1 ± 0.1 *	1.2 ± 0.1 *
5h	22 ± 0 *	1 ± 0 *	0.65 ± 0.01 *	0.84 ± 0.01 *

GSH	29 ± 1	0.69 ± 0.01	0.38 ± 0.02	0.53 ± 0.03

The kinetic parameters reveal that each selenohydantoin derivative exhibits a distinct behavior in terms of the time required to complete the reaction (TEC_{50}), the effectiveness of antioxidant activity (AE), as well as rate constants such as k_1 directly obtained from the slope of the non-linear *pseudo*-first order equation and k_2 derived from k_1 associated with the stoichiometric factor of the reaction. TEC_{50} is an initial kinetic parameter used to determine the reaction velocity in relation to the concentration of selenohydantoin derivatives (EC_{50}). Among the derivatives tested, **5b** with chlorine substitution had the best TEC_{50} value (11 ± 1 min) followed by **5e** (14 min ± 1) with -CF_3 substitution whilst **5a** with no substitution on the ring was the slowest to reach a steady state (42 min ± 1). The presence of a substitution group noticeably affects the velocity of the reaction in comparison with GSH.

Moreover, antiradical efficiency (AE), calculated upon kinetic (TEC_{50}) and akinetic (EC_{50}) values, was used to determine antioxidant efficiency allowing us to compare and classify selenohydantoin derivatives as follows:[45]

Low $AE \leq 1.0 \times 10^{-3}$

Medium $1.0 \times 10^{-3} < AE \leq 5.0 \times 10^{-3}$

High $5.0 \times 10^{-3} < AE \leq 10.0 \times 10^{-3}$

Therefore, most of the molecules fall into the medium class except **5a** and GSH, which, which belong to the low class. Based on their AE values, selenohydantoin derivatives can be ranked as follows **5b** > **5e** > **5g** > **5d** > **5h** > GSH > **5a.** This suggests that selenohydantoin derivatives are more effective than GSH. Moreover, their activity could be linked to various factors, such as selenium atom position in the molecule, tautomerism, the ease to engage in redox reactions, and the presence of the NH-bond, which can all contribute to the activity. Additionally, the presence of other substituents in the molecule, such as electron-withdrawing groups, may also play a role in their activity.

The evaluation of the antioxidant properties of selenohydantoin derivatives was also assessed with the FRAP assay. It is a non-radical antioxidant assay based on the ability of a molecule to donate an electron to reduce ferric ions (Fe^{3+}).[46,47] The FRAP values obtained for selenohydantoin derivatives, as well as the three control molecules, ascorbic acid, ebselen, and GSH, are represented in Table 4.

Table 4

Ferric Reducing Antioxidant Power (FRAP) values in μM for selenohydantoin derivatives and controls. Results are presented as mean ± sem, n = 3 and are compared using One-way ANOVA, and Bonferroni post-test, * $p < 0.05$ versus ascorbic acid, # $p < 0.05$ versus GSH

Compounds	FRAP value in μM
5a	219 ± 9 *, #
5b	421 ± 24 #
5c	180 ± 5 *, #
5d	439 ± 14 *, #
5e	363 ± 15 *, #
5f	116 ± 3 *
5g	314 ± 9 *, #
5h	271 ± 12 *, #
Ascorbic Acid	1632 ± 7 #
GSH	94 ± 1 *
Ebselen	No activity

The higher the FRAP value is, the higher the capacity of molecules to reduce ferric ions. Whereas ebselen, the benchmark for a selenium-containing GPx mimic, did not show any reducing activity regarding the FRAP assay, ascorbic acid exhibited the best FRAP value (1632 ± 7 μM). Selenohydantoin derivatives showed better FRAP values than GSH (94 ± 1 μM) and among these compounds, **5d** with a -F substituent exhibited the highest FRAP value (439 ± 14 μM). Comparing the results obtained from the FRAP and DPPH assays, it can be concluded that selenohydantoin derivatives are more efficient in radical scavenging than in one electron-donating capacity. For instance, **5e** carries a CF_3 group substitution on the benzene ring, which

is a highly electronegative and electron-withdrawing group due to the presence of three fluorine atoms. Therefore, the CF_3 group is polarized, with a partial negative charge on the fluorine atoms and a partial positive charge on the carbon atom. This makes the CF_3 group highly reactive in many chemical reactions. This strong electron-withdrawing ability can destabilize nearby chemical bonds, promote reactions and increase the electron-withdrawing ability of the benzene ring. Additionally, the partial positive charge on the carbon atom can be easly attacked by nucleophiles. The high electronegativity of the CF_3 group eases its leaving in substitution reactions. Indeed, the presence of fluorine atoms helps to stabilize the negative charge formed when the CF_3 group leaves the molecule. Interestingly, the CF_3 group further increases the acidity of any hydrogen atoms placed in the *ortho*- or *para*-positions in relation to the CF_3 group on the benzene ring. Indeed, the CF_3 group can contribute to stabilize the negative charge formed when the hydrogen is removed, making easier the disengagement of the hydrogen. This increased acidity can also increase the reactivity of molecules in in acid medium. Overall, the presence of a CF_3 group on a benzene ring significantly affects the reactivity and properties of the molecule, making it more electron-withdrawing and potentially more acidic impacting the type of reactions it undergoes.[48–50] Moreover, both DPPH and FRAP assays showed that **5b**, **5d**, **5e** and **5g** have greater antioxidant activity than the other of the selenohydantoin derivatives synthesized and also compared to GSH and ebselen.

The presence of a selenium atom in selenohydantoin derivatives may confer GPx-like activity to them, as seen in ebselen, the flagship molecule of the GPx mimetics, and selenocystamine, a commercially available diselenide. These molecules were employed as benchmark references for GPx-like activity to compare with our selenohydantoin derivatives and to investigate the importance of the catalytic cycle. Furthermore, it was observed that none of the selenohydantoin derivatives, nor ebselen, were capable of reducing *t*BuOOH.[51] However, selenohydantoin derivatives, ebselen along with selenocystamine showed a GPx-like activity in the presence of H_2O_2 (Figure 2) and cumene hydroperoxide (Figure 3).

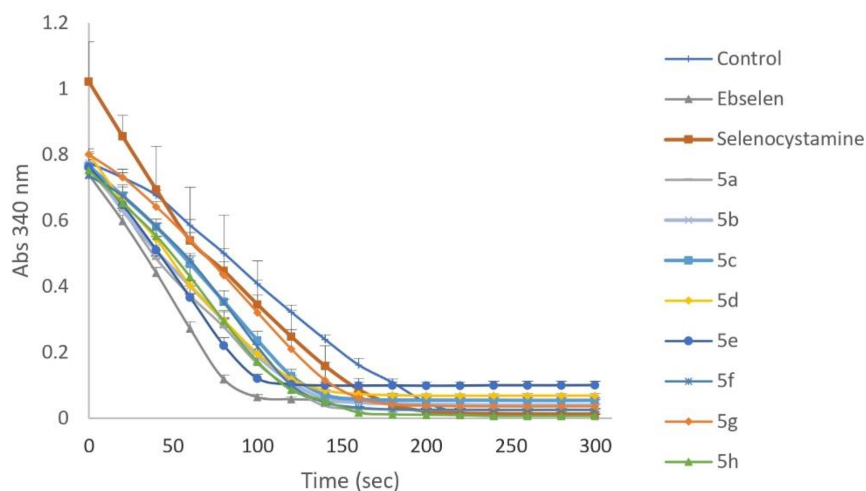


Figure 2. Kinetic curves of GPx-like activity in the presence of hydrogen peroxide (1 mM). The consumption of NADPH in the absence (control) or the presence of 20 µM ebselen or selenohydantoin derivatives was monitored at 340 nm. Results are presented as mean ± standard error of the mean, n = 3

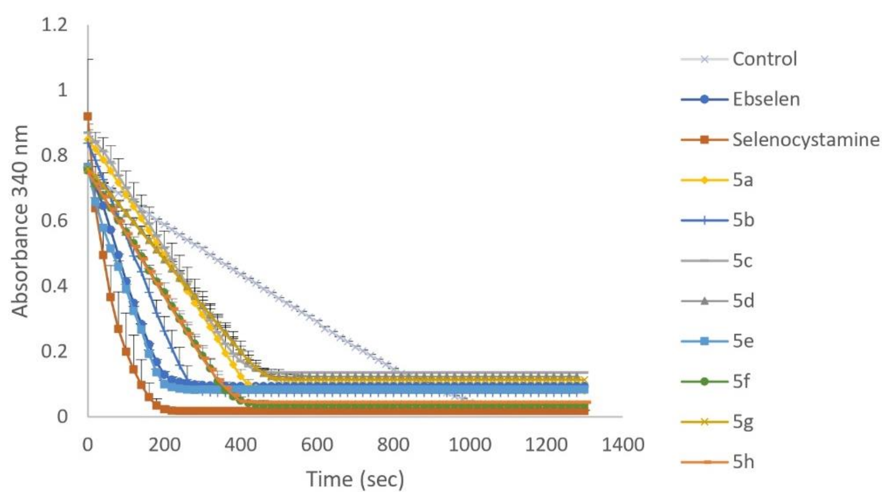


Figure 3. Kinetic curves of GPx-like activity in the presence of cumene hydroperoxide (1.5 mM). The consumption of NADPH (abs at 340 nm) was followed in the absence (control) or presence of 20 µM ebselen or selenohydantoin derivatives. Results are presented as mean ± sem, n=3

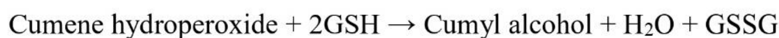
Table 5 shows the GPx-like activity of each compound. To calculate this activity, we used the linear part of enzymatic kinetic curves, as shown in Figure 2 and Figure 3. In addition, we determined the time required to complete the reaction as a parameter for evaluating the efficiency of the selenohydantoin derivatives.

Table 5

GPx-like activity and time needed to reach completion of the reaction. Those parameters were determined by monitoring the disappearance of NADPH (Abs at 340 nm) in the absence (control) or the presence of selenohydantoin derivatives, ebselen or selenocystamine at 20 µM. Results are presented as mean ± sem, n = 3 and are compared using One-way ANOVA, and Bonferroni post-test, * $p < 0.05$ *versus* control and # $p < 0.05$ *versus* ebselen

	GPx-like activity (µM NADPH min^{-1})		Time to reach completion of the linear part (s)	
Compounds	**H_2O_2**	**Cumene hydroperoxide**	**H_2O_2**	**Cumene hydroperoxide**
5a	55 ± 4 #, *	18 ± 1 #, *	113 ± 12	413 ± 12
5b	54 ± 6 #, *	27 ± 2 #, *	113 ± 12	253 ± 12
5c	51 ± 2 #, *	17 ± 0 #, *	127 ± 12	393 ± 12
5d	57 ± 2 #, *	13 ± 0 #, *	107 ± 12	460 ± 20
5e	**63 ± 1** #, *	**32 ± 1** *	**100 ± 0**	**180 ± 0**
5f	55 ± 3 #, *	18 ± 1 #, *	107 ± 12 (From 20 to 127)	367 ± 12
5g	49 ± 5 #, *	13 ± 1 #, *	140 ± 12 (From 7 to 147)	467 ± 23
5h	39 ± 1 #	18 ± 2	187 ± 12	360 ± 40
Control	37 ± 1 #	7 ± 1 #	200 ± 0	907 ± 12
Ebselen	75 ± 1 *	33 ± 1 *	80 ± 0	187 ± 12
Selenocystamine	53 ± 5 #, *	66 ± 7 #, *	153 ± 12	93 ± 12

Each selenohydantoin derivative showed a GPx-like activity higher with H_2O_2 than with cumene hydroperoxide, which is often employed to evaluate the activity of Se and non-Se enzymes to reduce organic peroxide, in addition, H_2O_2 has a higher affinity for GPx-like enzymes compared to cumene hydroperoxide, due to its small size, and uncharged molecule that can easily interact with the catalytic part of the enzyme. While, cumene hydroperoxide is a larger, less stable, and more complex molecule so it can be more difficult to interact with the active site of the enzyme, and therefore react in a slower manner.[52–55] This suggests that selenohydantoin derivatives exhibit GPx-like activity by reducing hydroperoxides through a Se-dependent reaction and organic peroxides through a non-Se-dependent activity that was observed when cumene hydroperoxide was employed. Cumene hydroperoxide is known to be a substrate that evaluates GPx-like activity since it is an organic peroxide that can be reduced by molecules in a reaction that is catalyzed by GPx enzymes. However, unlike other peroxides such as hydrogen peroxide (H_2O_2), cumene hydroperoxide can be reduced by molecules even in the absence of selenium, suggesting a non-selenium-dependent mechanism of action following the reaction:[56–59]



Where Cumene hydroperoxide is reduced to cumyl alcohol, and two molecules of GSH are oxidized to form oxidized glutathione (GSSG).

Moreover, selenohydantoin derivatives have lower GPx-like activity than ebselen, except **5e** exhibiting the same activity as ebselen with both hydrogen peroxide and cumene hydroperoxide. It is worth noting that their non-Se dependent GPx-like activity with cumene hydroperoxide was significantly lower than with H_2O_2, particularly when compared to selenocystamine. Notably, except for **5e**, the time to complete the linear part of the reaction was longer for each selenohydantoin derivative than for ebselen whereas this reaction was also generally faster compared to selenocystamine. **5f** and **5g** exhibited a delay of 20 s and 7 s respectively, in starting the reaction.

Based on previous findings, it is clear that there is a relationship between activity and structure, which is not surprising given that each molecule has two different rings and different groups attached to them. Moreover, the selenium and the oxygen atoms attached to the heterocycle withdraw electrons

from the ring. They are in a state where selenium, for instance, can potentially undergo tautomerism resulting in the presence of the selenone, selenol, and selenolate species. These species each play a role in antioxidant activity in general and in GPx-like activity specifically.[60–62] Furthermore, it was observed that the molecules exhibited varying activities, which could be linked directly to differences in solubility and stability, as well as to the substituents on the aryl group. For instance, **5e**, which contained a trifluoromethyl group, demonstrated significant activity in most of the tests. This could be attributed as already stated to the electron-withdrawing effect of the $-CF_3$ group. The fluorine atoms withdraw electron density from the carbon atom to which they are directly attached, thereby inducing an inductive effect that withdraws electron density from the ring. This suggests the ability of the CF_3 group to stabilize the resulting selenenyl sulfide intermediate during the GPx catalytic cycle. The CF_3 group can be effective in stabilizing the intermediate, leading to enhanced GPx-like activity.

To gain a better understanding of whether the existence of more than one tautomer of selenohydantoin derivatives in solution is viable, we performed theoretical calculations using Density Functional Theory (DFT). Specifically, we evaluated the relative stability of the eight possible tautomers of selenohydantoin derivative **5a** (shown in Figure 4). For this analysis, we selected **5a** as a model of selenohydantoin derivative without substitution, assuming that substitution would not affect tautomerism. Calculations and structural details are presented in supplementary S24 to S51. Interestingly, results revealed that only the selenone isomer **A** should occur in water, with the Gibbs distribution indicating that more than 99.99% of the molecules should be in this isomeric form. This profile was also observed when using methanol and DMSO as solvents. These results pointed out that selenol isomers (**B**, **C**, **E**, **G,** and **H**) are probably not involved in the antioxidant properties of selenohydantoin derivatives **5a-5h**. Moreover, the occurrence of keto-enol tautomerism involving the carbon at the 5-position of the selenohydantoin ring is also not probable to occur, even when it results in the formation of an aromatic ring (*e.g.* **G** and **H**).

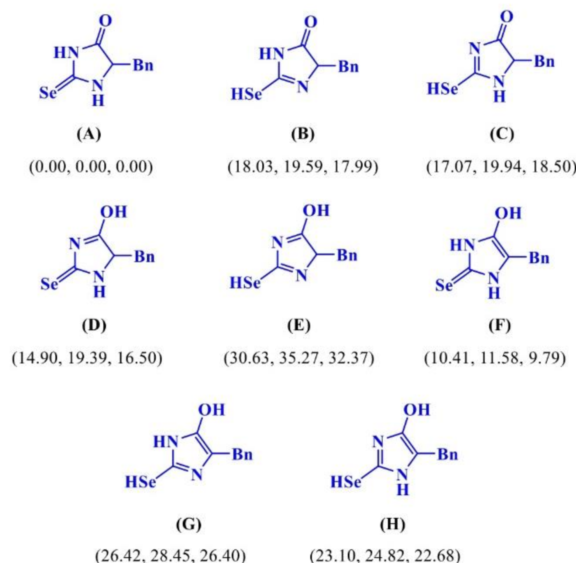


Figure 4. Hypothetical structures of the eight possible isomers of compound **5a**. In brackets are shown the values of the relative energies (in kcal mol^{-1}) at 310.15 K of these species in water, methanol, and dimethylsulfoxide, respectively.

The presence of a basic species, such as phosphate, carbonate, or sulfate, could potentially result in the formation of a selenolate form, particularly since hydantoins have a highly acidic proton.[63] In light of this, we chose to use phosphate buffer as a model for our calculations since it was employed in some of the experimental procedures. Specifically, we evaluated the proton transfer of compound **5a** to phosphate and found that it occurred barrierless in water at 310.15 K. Thus, the relative distribution of selenone/selenolate forms is determined only by the relative stabilities of these species ($\Delta G = 1.31$ kcal mol^{-1}) leading to 89.34% of selenone and 10.66% of selenolate according to the Gibbs distribution. Considering this, the selenolate might be involved in the antioxidant activity of selenohydantoin derivatives **5a-5h**.

These encouraging results have paved the way for further biological evaluations. To ensure the success of these assessments, we first evaluated the cytocompatibility of the selenohydantoin derivatives. For this purpose, the metabolic activity of HuAoSMC was evaluated after 24 h of incubation with selenohydantoin derivatives from 1 μM to 100 μM and was compared to selenocystamine (Figure 5). Indeed, ebselen could not be tested as it was impossible to solubilize it sufficiently in the culture medium containing 0.1% DMSO (maximum DMSO concentration tolerated by HuAoSMC) due to its high cLog*P* value.

According to a study, when human aortic smooth muscle cells (HuAoSMC) were incubated with selenohydantoin derivatives at concentrations below 100 µM, their metabolic activity was over 80%, which is considered the limit point for cell viability. This finding suggests that the selenohydantoin derivatives are cytocompatible. In contrast, the study found that selenocystamine was toxic even at a concentration of 1 µM. This crucial information will guide us in designing further experimental work and unlocking the full potential of these selenohydantoin derivatives.

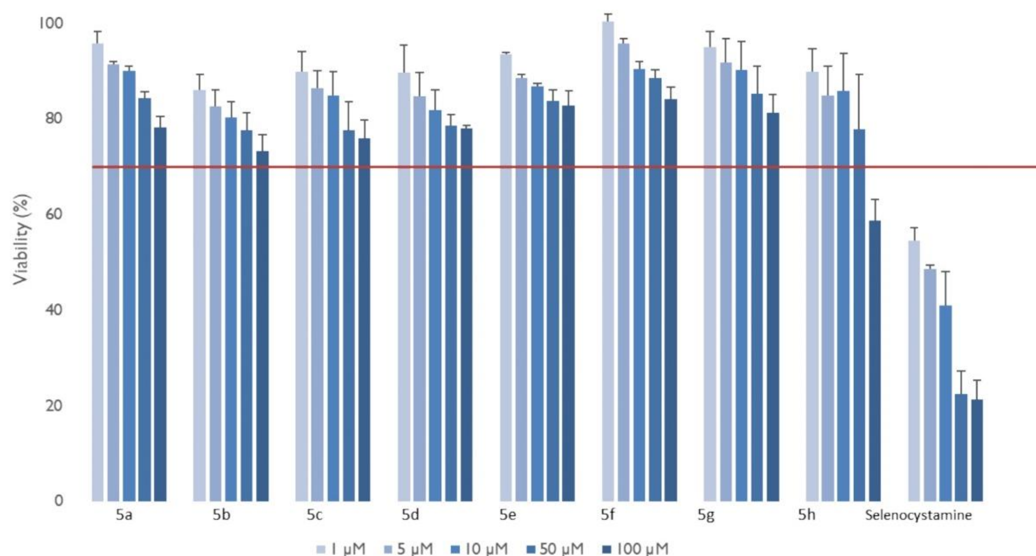


Figure 5. Cytocompatibility of selenohydantoin derivatives with HuAoSMC. Selenohydantoin derivatives or selenocystamine at 1 µM, 5 µM, 10 µM, 50 µM, and 100 µM were incubated for 24 h with cells followed by MTT assay. Results are presented as mean ± sem, n = 3 in triplicate.

3. Materials and Methods

All commercially available reagents were purchased from Sigma-Aldrich or Alfa Aesar and used as received. The melting points were taken on Electrothermal IA9000 digital melting point apparatus and are reported uncorrected. Hydrogen nuclear magnetic resonance (^{1}H NMR) spectra, carbon nuclear magnetic resonance (^{13}C NMR) spectra and Selenium nuclear magnetic resonance (^{77}Se) were recorded on a Bruker Variance 500 MHz spectrometer. Chemical shifts are reported in parts per million in DMSO-*d6*. Data are represented as follows: chemical shift, multiplicity (br = broad, s = singlet, d = doublet, t = triplet, q = quartet, quint = quintet, sextet = sext, heptet = hept, octet = oct, dd = doublet of doublets, dt = doublet of triplets, m = multiplet), coupling constants in hertz (Hz), integration. The

High-resolution mass spectra were obtained on a 7 Tesla Solarix FT-ICR-MS (Brucker Daltonics) equipped with an electrospray ionization source, applied in negative and positive mode. Samples were infused into the electrospray ionization source (ESI) at a flow rate 2 uL/min in negative ion mode. Dry gas 4.0 L/min, dry temp 200 C, nebulizer 1 bar. The lower and upper mass limit was set between m/z 150 and 1000. Spray shield voltage was set to 2.5 kV, the capillary voltage was set to 3 kV, and the capillary column end voltage was 4200 V. Chromatographic purifications were conducted by column chromatography using silica gel (230−400 mesh, 40−63μm), eluting with hexane or hexane:EtOAc mixtures of increasing polarity. Progress of the reactions was monitored by thin layer chromatography, using UV-light (254 and 365nm), I_2 or H_2SO_4/vanillin solution for detection of the spots.

3.1. Synthesis of selenohydantoin derivatives

Several synthetic methods for the Knoevenagel condensation reaction and the *S*-alkylation reaction for hydantoin derivative synthesis (**3** and **4**) are described in the literature. However, there are no previous reports on the synthesis of 5-aryl-2-selenoxoimidazolidin-4-ones derivatives **5**.[64–73] Considering this, we propose a synthetic route for obtaining derivatives of **5** through a selenylation reaction followed by reduction of the sp^2 carbon of the respective derivatives **4**. Structures of selenohydantoin derivatives were determined by ^{1}H NMR, ^{13}C NMR, ^{77}Se NMR, and HRMS.

General Procedure for the Preparation of the (*Z*)-5-arylidene-2-thioxoimidazolidin-4-ones (3a–3h).[72]

In a round-bottom flask equipped with a magnetic stirrer and condenser, thiohydantoin (5.0 mmols) and the respective aromatic aldehyde (6.0 mmols, 1.2 equiv.) were added in water (5 mL), followed by 15 drops of piperidine. The reaction solution was heated to 90 °C and maintained under rigorous magnetic stirring overnight. After, the reaction system was cooled in an ice bath and the precipitate was filtered under vacuum, being purified by washing with diethyl ether and dried under reduced pressure.

(*Z*)-5-benzylidene-2-thioxoimidazolidin-4-one (3a)

Yellow solid (745 mg, 3.64 mmol, 73% yield): ^{1}H NMR (400 MHz, DMSO-*d6*): δ 12.26 (s, 2H), 7.75–7.73 (m, 2H), 7.44–7.36 (m, 3H), 6.48 (s, 1H). ^{13}C{^{1}H} NMR (100 MHz, DMSO-*d6*) δ 179.3, 165.8, 132.3, 130.1, 129.2, 128.7, 127.7, 111.5.

(*Z*)-5-(4-chlorobenzylidene)-2-thioxoimidazolidin-4-one (3b)

Yellow solid (1020 mg, 4.25 mmol, 85% yield): ^{1}H NMR (400 MHz, DMSO-*d6*): δ 12.21 (s, 2H), 7.76 (d, *J* = 8.5, 2H), 7.45 (d, *J* = 8.5 Hz, 2H), 6.45 (s, 1H). ^{13}C{^{1}H} NMR (100 MHz, DMSO-*d6*) δ 179.4, 165.8, 133.6, 131.7, 131.4, 128.7, 128.5, 109.8.

(*Z*)-5-(4-bromobenzylidene)-2-thioxoimidazolidin-4-one (3c)

Yellow solid (1160 mg, 4.1 mmol, 82% yield): ^{1}H NMR (400 MHz, DMSO-*d6*): δ 12.38 (s, 1H), 12.21 (s, 1H), 7.69–7.68 (m, 2H), 7.60–7.58 (m, 2H), 6.44 (s, 1H). ^{13}C{^{1}H} NMR (100 MHz, DMSO-*d6*) δ 179.44, 165.75, 132.01, 131.71, 131.65, 128.27, 122.56, 110.05.

(*Z*)-5-(4-fluorobenzylidene)-2-thioxoimidazolidin-4-one (3d)

Yellow solid (1010 mg, 4.5 mmol, 91% yield): ^{1}H NMR (400 MHz, DMSO-*d6*): δ 12.18 (s, 2H), 7.82–7.78 (m, 2H), 7.26–7.22 (m, 2H), 6.48 (s, 1H). ^{13}C{^{1}H} NMR (100 MHz, DMSO-*d6*) δ 179.3, 165.8, 162.3 (d, $J_{C\text{-}F}$ = 248.6 Hz), 132.5 (d, $J_{C\text{-}F}$ = 8.3 Hz), 129 (d, $J_{C\text{-}F}$ = 2.9 Hz), 127.8, 115.8 (d, $J_{C\text{-}F}$ = 21.7 Hz), 110.3.

(*Z*)-2-thioxo-5-(4-(trifluoromethyl)benzylidene)imidazolidin-4-one (3e)

Yellow solid (1225 mg, 4.5 mmol, 90% yield): ^{1}H NMR (400 MHz, DMSO-*d6*): δ 12.20 (s, 2H), 7.94 (d, *J* = 8.2 Hz, 2H), 7.74 (d, *J* = 8.2 Hz, 2H), 6.50 (s, 1H). ^{13}C{^{1}H} NMR (100 MHz, DMSO-*d6*) δ 180.1, 166.1, 136.8, 130.6, 130.5, 128.3 (q, $J_{C\text{-}F}$ = 31.8), 125.3 (q, $J_{C\text{-}F}$ = 3.6), 124.1 (q, $J_{C\text{-}F}$ = 272.0), 108.7.

(*Z*)-5-(4-methylbenzylidene)-2-thioxoimidazolidin-4-one (3f)

Yellow solid (709 mg, 3.25 mmol, 65% yield): ^{1}H NMR (400 MHz, DMSO-*d6*): δ 12.34 (s, 1H), 12.09 (s, 1H), 7.65 (d, *J* = 8.2 Hz, 2H), 7.24 (d, *J* = 8.0 Hz, 2H), 6.45 (s, 1H), 2.33 (s, 3H). ^{13}C{^{1}H} NMR (100 MHz, DMSO-*d6*) δ 178.9, 165.8, 139.3, 130.2, 129.5, 129.4, 126.9, 111.9, 21.0.

(*Z*)-5-(4-methoxybenzylidene)-2-thioxoimidazolidin-4-one (3g)

Yellow solid (890 mg, 3.8 mmol, 76% yield): ^{1}H NMR (400 MHz, DMSO-*d6*): δ 12.09 (s, 2H), 7.75–7.72 (m, 2H), 6.99–6.96 (m, 2H), 6.46 (s, 1H), 3.81 (s, 3H). ^{13}C{^{1}H} NMR (100 MHz, DMSO-*d6*) δ 178.6, 165.9, 160.2, 132.1, 126.0, 124.9, 114.3, 112.1, 55.3.

General Procedure for the Preparation of the (*Z*)-5-arylidene-2-(methylthio)-3,5-dihydro-4*H*-imidazol-4-ones (4a–4h).[71]

In a round-bottom flask equipped with a magnetic stirrer and condenser was added successively the (*Z*)-5-arylidene-2-thioxoimidazolidin-4-one (2.0 mmol), 5 mL of acetonitrile and K_2CO_3 (1.0 mmol, 0.5 equiv.). To this suspension was added iodomethane (3.0 mmol, 1.5 equiv.) and the resulting reaction mixture was heated to 40 °C keeping overnight. After cooling down to room temperature, 5 mL of water was added in the crude suspension. The insoluble product was collected by filtration and washed successively with water (5 mL) and diethyl ether (3 mL). The desired product was purified by silica gel chromatography (eluent hexane/AcOEt 90:10).

(*Z*)-5-benzylidene-2-(methylthio)-1,5-dihydro-4H-imidazol-4-one (4a)

Yellow solid (405 mg, 1.83 mmol, 93% yield): ^{1}H NMR (400 MHz, DMSO-*d6*): δ 8.17 (d, *J* = 7.3 Hz, 2H), 7.44–7.41 (m, 2H), 7.38–7.35 (m, 1H), 6.71 (s, 1H), 3.44 (bs, 1H), 2.66 (s, 3H). ^{13}C{^{1}H} NMR (100 MHz, DMSO-*d6*) δ 171.2, 166.0, 139.5, 134.4, 131.3, 129.3, 128.5, 120.2, 12.2.

(*Z*)-5-(4-chlorobenzylidene)-2-(methylthio)-1,5-dihydro-4H-imidazol-4-one (4b)

Yellow solid (480 mg, 1.9 mmol, 95% yield): ^{1}H NMR (400 MHz, DMSO-*d6*): δ 8.18–8.17 (m, 2H), 7.46–7.45 (m, 2H), 6.60 (s, 1H), 4.96 (bs, 1H), 2.63 (s, 3H).

(*Z*)-5-(4-bromobenzylidene)-2-(methylthio)-1,5-dihydro-4H-imidazol-4-one (4c)

Yellow solid (528 mg, 1.78 mmol, 89% yield): ^{1}H NMR (400 MHz, DMSO-*d6*): δ 8.13–8.11 (m, 2H), 7.63–7.61 (m, 2H), 6.68 (s, 1H), 3.40 (bs, 1H), 2.65 (s, 3H). ^{13}C{^{1}H} NMR (100 MHz, DMSO-*d6*) δ 171.3, 167.1, 140.2, 133.7, 133.0, 131.6, 122.6, 118.5, 12.2.

(*Z*)-5-(4-fluorobenzylidene)-2-(methylthio)-1,5-dihydro-4H-imidazol-4-one (4d)

Yellow solid (439 mg, 1.86 mmol, 93% yield): ^{1}H NMR (400 MHz, DMSO-*d6*): δ 8.24–8.20 (m, 2H), 7.25–7.21 (m, 2H), 6.59 (s, 1H), 4.46 (bs, 1H), 2.61 (s, 3H). ^{13}C{^{1}H} NMR (100 MHz,

DMSO-*d6*) δ 173.9, 170.2, 162.0 (d, J_{C-F} = 247.7), 140.8, 133.2 (d, J_{C-F} = 8.1 Hz), 131.7 (d, J_{C-F} = 3.0 Hz), 117.0, 115.5 (d, J_{C-F} = 21.3 Hz), 12.3.

(*Z*)-2-(methylthio)-5-(4-(trifluoromethyl)benzylidene)-1,5-dihydro-4H-imidazol-4-one (4e)

Yellow solid (475 mg, 1.66 mmol, 83% yield): ^{1}H NMR (400 MHz, DMSO-*d6*): δ 11.95 (bs, 1H), 8.38 (d, *J* = 8.2 Hz, 2H), 7.77 (d, *J* = 8.2 Hz, 2H), 6.77 (s, 1H), 2.68 (s, 3H). ^{13}C{^{1}H} NMR (100 MHz, DMSO-*d6*) δ 170.9, 167.9, 141.2, 138.4, 131.6, 128.8 (q, J_{C-F} = 31.8 Hz), 125.2 (q, J_{C-F} = 3.8 Hz), 124.0 (q, J_{C-F} = 272.4 Hz), 117.8, 12.3.

(*Z*)-5-(4-methylbenzylidene)-2-(methylthio)-1,5-dihydro-4H-imidazol-4-one (4f)

Yellow solid (427 mg, 1.84 mmol, 92% yield): ^{1}H NMR (400 MHz, DMSO-*d6*): δ 11.76 (bs, 1H), 8.06 (d, *J* = 8.1 Hz, 2H), 7.24 (d, *J* = 8.1 Hz, 2H), 6.68 (s, 1H), 2.65 (s, 3H), 2.33 (s, 3H). ^{13}C{^{1}H} NMR (100 MHz, DMSO-*d6*) δ 171.4, 165.4, 139.2, 138.9, 131.7, 131.4, 129.2, 120.4, 21.1, 12.2.

(*Z*)-5-(4-methoxybenzylidene)-2-(methylthio)-1,5-dihydro-4H-imidazol-4-one (4g)

Yellow solid (407 mg, 1.64 mmol, 82% yield): ^{1}H NMR (400 MHz, DMSO-*d6*): δ 11.65 (bs, 1H), 8.16 (d, *J* = 8.9 Hz, 2H), 7.00 (d, *J* = 8.9 Hz, 2H), 6.71 (s, 1H), 3.80 (s, 3H), 2.65 (s, 3H). ^{13}C{^{1}H} NMR (100 MHz, DMSO-*d6*) δ 170.8, 163.5, 160.3, 137.5, 133.3, 127.0, 120.9, 114.2, 55.2, 12.1.

(*Z*)-2-(methylthio)-5-(naphthalen-1-ylmethylene)-1,5-dihydro-4H-imidazol-4-one (4h)

Yellow solid (456 mg, 1.7 mmol, 85% yield): ^{1}H NMR (400 MHz, DMSO-*d6*): δ 8.45 (dd, *J* = 8.5, 1.5 Hz, 1H), 8.43 (br, 1H), 7.87–7.84 (m, 3H), 7.48–7.46 (m, 2H), 6.43 (s, 1H), 2.59 (s, 3H).

General Procedure for the Preparation of the 5-aryl-2-selenoxoimidazolidin-4-ones (5a–5h).[36]

In a round-bottom flask equipped with a magnetic stirrer and condenser, the Se° (5.0 mmol) in ethanol (20 mL) was added successively followed by the addition of $NaBH_4$ (10.0 mmol, 2 equiv.). The mixture was stirred under argon until complete consumption of selenium (colour-

less solution). Then, the respective **4** (1.0 mmol) was added in a single portion at room temperature. Afterwards, the system was raised to the reflux temperature, and kept under magnetic stirrer for 8 hours. After cooling down to room temperature, the reaction mixture was directly filtered through a sintered funnel containing silica gel, being eluted with a 1:1 solution of ethyl acetate and hexane. The combined organic phases were dried over $MgSO_4$, filtered and the solvent was eliminated in a rotary evaporator under reduced pressure, providing the desired product.

5-benzyl-2-selenoxoimidazolidin-4-one (5a)

White solid (180 mg, 0.71 mmol, 71% yield): mp 190.8 °C. ^{1}H NMR (400 MHz, DMSO-*d6*): δ 11.77 (s, 1H), 10.75 (s, 1H), 7.29–7.25 (m, 2H), 7.24–7.21 (m, 1H), 7.17–7,15 (m, 1H), 4.44–4.42 (m, 1H), 3.02 (dd, J = 14.2, 5.1 Hz, 1H), 2.97 (dd, J = 14.2, 4.8 Hz, 1H). ^{13}C{^{1}H} NMR (100 MHz, DMSO-*d6*) δ 180.1, 175.6, 134.7, 129.5, 128.1, 126.8, 62.3, 35.0. ^{77}Se NMR (76 MHz, DMSO-*d6*) δ 224.3. HRMS (ESI) m/z: $[M + Na]^+$ calcd for $C_{10}H_{10}N_2OSe$: 276.98504; found: 276.98790.

5-(4-chlorobenzyl)-2-selenoxoimidazolidin-4-one (5b)

White solid (187 mg, 0.65 mmol, 65% yield): mp 196.1 °C. ^{1}H NMR (400 MHz, DMSO-*d6*): δ 11.82 (s, 1H), 10.76 (s, 1H), 7.36–7.33 (m, 2H), 7.19–7.16 (m, 2H), 4.45–4.43 (m, 1H), 3.01 (dd, J = 14.2, 5.1 Hz, 1H), 2.97 (dd, J = 14.2, 5.0 Hz, 1H). ^{13}C{^{1}H} NMR (100 MHz, DMSO-*d6*) δ 180.3, 175.5, 133.8, 131.7, 131.4, 128.1, 62.1, 34.3. ^{77}Se NMR (76 MHz, DMSO-*d6*) δ 226.4. HRMS (ESI) m/z: $[M + Na]^+$ calcd for $C_{10}H_9ClN_2OSe$: 310.94607; found: 310.94795.

5-(4-bromobenzyl)-2-selenoxoimidazolidin-4-one (5c)

Yellowish solid (209 mg, 0.63 mmol, 63% yield): mp 197.2 °C. ^{1}H NMR (400 MHz, DMSO-*d6*): δ 11.84 (s, 1H), 10.76 (s, 1H), 7.49–7.46 (m, 2H), 7.13–7.10 (m, 2H), 4.45–4.43 (m, 1H), 2.99 (dd, J = 14.2, 5.1 Hz, 1H), 2.95 (dd, J = 14.2, 5.0 Hz, 1H). ^{13}C{^{1}H} NMR (100 MHz, DMSO-*d6*) δ 180.3, 175.5, 134.2, 131.8, 131.0, 120.2, 62.1, 34.4. ^{77}Se NMR (76 MHz, DMSO-*d6*) δ 226.8. HRMS (ESI) m/z: $[M + Na]^+$ calcd for $C_{10}H_9BrN_2OSe$: 354.89555; found: 354.89734.

5-(4-fluorobenzyl)-2-selenoxoimidazolidin-4-one (5d)

Yellowish solid (184 mg, 0.68 mmol, 68% yield): mp 200 °C. ^{1}H NMR (400 MHz, DMSO-*d6*): δ 11.81 (s, 1H), 10.76 (s, 1H), 7.21–7.17 (m, 2H), 7.13–7.08 (m, 2H), 4.43–4.41 (m, 1H), 3.01 (dd, J = 14.4, 5.2 Hz, 1H), 2.97 (dd, J = 14.4, 5.0 Hz, 1H). ^{13}C{^{1}H} NMR (100 MHz, DMSO-*d6*) δ 180.2, 175.6, 161.3 (d, $J_{C\text{-}F}$ = 242.3 Hz), 131.4 (d, $J_{C\text{-}F}$ = 8.1 Hz), 130.9 (d, $J_{C\text{-}F}$ = 2.8 Hz), 114.9 (d, $J_{C\text{-}F}$ = 21.1 Hz), 62.3, 34.2. ^{77}Se NMR (76 MHz, DMSO-*d6*) δ 225.1. HRMS (ESI) m/z: $[M + Na]^+$ calcd for $C_{10}H_9FN_2OSe$: 294.97562; found: 294.98540.

2-selenoxo-5-(4-(trifluoromethyl)benzyl)imidazolidin-4-one (5e)

Yellowish solid (231 mg, 0.72 mmol, 72% yield): mp 209 °C. ^{1}H NMR (400 MHz, DMSO-*d6*): δ 11.88 (s, 1H), 10.80 (s, 1H), 7.66 (d, J = 8.0 Hz, 2H), 7.40 (d, J = 8.0 Hz, 2H), 4.51 – 4.49 (m, 1H), 3.12 (dd, J = 14.1, 5.2 Hz, 1H), 3.06 (dd, J = 14.1, 5.3 Hz, 1H). ^{13}C{^{1}H} NMR (100 MHz, DMSO-*d6*) δ 180.4, 175.5, 139.9, 130.4, 127.6 (q, $J_{C\text{-}F}$ = 31.5 Hz), 125.0 (q, $J_{C\text{-}F}$ = 3.4 Hz), 124.3 (q, $J_{C\text{-}F}$ = 272.1 Hz), 62.0, 34.9. ^{77}Se NMR (76 MHz, DMSO-*d6*) δ 228.4. HRMS (ESI) m/z: $[M + Na]^+$ calcd for $C_{11}H_9F_3N_2OSe$: 344.97242; found: 344.99188.

5-(4-methylbenzyl)-2-selenoxoimidazolidin-4-one (5f)

White solid (176 mg, 0.66 mmol, 66% yield): mp 204.1 °C. ^{1}H NMR (400 MHz, DMSO-*d6*): δ 11.74 (s, 1H), 10.74 (s, 1H), 7.08 – 7.06 (m, 2H), 7.05 – 7.03 (m, 2H), 4.41 – 4.39 (m, 1H), 2.99 – 2.91 (m, 2H), 2.25 (s, 3H). ^{13}C{^{1}H} NMR (100 MHz, DMSO-*d6*) δ 180.1, 175.7, 135.9, 131.6, 129.5, 128.7, 62.4, 34.5, 20.6. ^{77}Se NMR (76 MHz, DMSO-*d6*) δ 223.9. HRMS (ESI) m/z: $[M + Na]^+$ calcd for $C_{11}H_{12}N_2OSe$: 291.00069; found: 291.00141.

5-(4-methoxybenzyl)-2-selenoxoimidazolidin-4-one (5g)

Yellow solid (224 mg, 0.79 mmol, 79% yield): mp 210.7 °C. ^{1}H NMR (400 MHz, DMSO-*d6*): δ 11.74 (s, 1H), 10.72 (s, 1H), 7.08–7.05 (m, 2H), 6.84–6.81 (m, 2H), 4.38–4.36 (m, 1H), 3.71 (s, 3H), 2.95 (dd, J = 14.2, 4.8 Hz, 1H), 2.91 (dd, J = 14.2, 4.6 Hz, 1H). ^{13}C{^{1}H} NMR (100 MHz, DMSO-*d6*) δ 180.1, 175.7, 158.2, 130.6, 126.5, 113.5, 62.5, 54.9, 34.1. ^{77}Se NMR (76 MHz, DMSO-*d6*) δ 223.4. HRMS (ESI) m/z: $[M + Na]^+$ calcd for $C_{11}H_{12}N_2O_2Se$: 306.9956; found: 306.99621.

5-(naphthalen-1-ylmethyl)-2-selenoxoimidazolidin-4-one (5h)

White solid (212 mg, 0.7 mmol, 70% yield): mp 204 °C. ^{1}H NMR (400 MHz, DMSO-*d6*): δ 11.78 (s, 1H), 10.84 (s, 1H), 7.88–7.86 (m, 1H), 7.84–7.82 (m, 2H), 7.68 (s, 1H), 7.51–7.46

(m, 2H), 7.35 (dd, J = 8.4, 1.7 Hz, 1H), 4.53 (dd, J = 5.2, 5.0 Hz, 1H), 3.20 (dd, J = 14.2, 5.0 Hz, 1H), 3.14 (dd, J = 14.2, 5.2 Hz, 1H). $^{13}C\{^{1}H\}$ NMR (100 MHz, DMSO-*d6*) δ 180.2, 175.7, 132.8, 132.6, 132.0, 128.1, 127.8, 127.6, 127.5 (x2), 126.1, 125.7, 62.4, 35.3. ^{77}Se NMR (76 MHz, DMSO-*d6*) δ 226.1. HRMS (ESI) m/z: $[M + Na]^+$ calcd for $C_{14}H_{12}N_2OSe$: 327.00069; found: 327.00165.

3.2. Physicochemical characterization of selenohydantoin derivatives

Melting points of selenohydantoin derivatives were obtained on an Electrothermal IA9000 digital melting point apparatus and are uncorrected. Hydrogen nuclear magnetic resonance (^{1}H NMR) spectra, carbon nuclear magnetic resonance (^{13}C NMR) spectra, and selenium nuclear magnetic resonance (^{77}Se) were recorded on a Bruker Variance 500 MHz spectrometer. Chemical shifts are reported in parts per million in DMSO-*d6*. Data are represented as follows: chemical shift, multiplicity (br = broad, s = singlet, d = doublet, t = triplet, q = quartet, quint = quintet, sextet = sext, heptet = hept, octet = oct, dd = doublet of doublets, dt = doublet of triplets, m = multiplet), coupling constants in Hertz (Hz). The High-resolution mass spectra were obtained on a 7 Tesla Solarix FT-ICR-MS (Brucker Daltonics) coupled with an electrospray ionization source, applied in negative and positive modes. Samples were dissolved in methanol and infused into the electrospray ionization source (ESI) at a flow rate of 5 μL min^{-1} *via* a direct infusion. Dry gas 4.0 L min^{-1}, dry temperature 200 °C, nebulizer 1 bar. The lower and upper mass limits were set between 150 and 1000 m z^{-1}. The spray shield voltage was set to 2.5 kV. The capillary voltage was set to 3 kV, and the capillary column end voltage was 4200 V. Records were treated with Data Analysis software using the Smart Formula option to obtain the molecular formula. Chromatographic purifications were conducted by column chromatography using silica gel (230−400 mesh, 40−63 μm) and eluted with hexane or hexane:EtOAc mixtures of increasing polarity. Progress of the reactions was monitored by thin layer chromatography, using UV-light (254 and 365 nm), and I_2 or H_2SO_4/vanillin solution for visualization of the spots.

Calculated log*P* (cLog*P*) values were obtained with ChemDraw 15.0 software (specific algorithms for calculating log*P* from fragment-based methods were developed by the Medicinal

Chemistry Project of CambridgeSoft and BioByte). The cLog*P* value of a compound is the logarithmic partition coefficient between *n*-octanol and water, log(c(octanol) /c (water)), and determines the place of a molecule on the lipophile/hydrophile balance.[74] The maximum peak of absorbance (λ_{max} Abs) was also determined for selenium derivatives at a concentration of 50 µM using a Shimadzu UV-VIS spectrophotometer (UV-2600) to avoid any potential interaction with the subsequent spectrophotometric assays.

3.3. Radical scavenging capacity

3.3.1. Akinetic mode

The 1,1'-diphenyl-2-picrylhydrazyl radical ($DPPH^{\bullet}$) is a stable nitrogen-centered radical, which has a maximal absorbance peak at λ= 515 nm (ε = 12.5 mM^{-1} cm^{-1}) (deep violet color). The fading of the violet color in the presence of antioxidants indicates the reduction of $DPPH^{\bullet}$ to DPPH-H and is monitored at 515 nm using a Shimadzu UV-VIS spectrophotometer (UV-2600). This is employed to evaluate direct radical scavenging properties. In this assay, antioxidants reduce $DPPH^{\bullet}$ either by Hydrogen Atom Transfer (HAT) or Single Electron Transfer (SET). Selenohydantoin derivatives (7 µM to 57 µM) or control molecules (ascorbic acid, GSH, or ebselen) were mixed with 71 µM $DPPH^{\bullet}$ in methanol–ammonium citrate buffer 10 mM pH 7.4 (60:40, v/v), and then incubated for ten minutes in the dark before reading the absorbance.[43] The half maximal efficient concentration (EC_{50}) values were calculated for each compound from the slope of the linear part (y = mx + b), whilst the stoichiometric coefficient was calculated as the ratio between the antioxidant EC_{50} value in µM and the half of DPPH initial concentration (35.5 µM). This stoichiometric coefficient represents the number of selenohydantoin molecules reacting with one DPPH molecule.

3.3.2. Kinetic mode

To determine the kinetic parameters of the reaction, the reduction of the $DPPH^{\bullet}$ radical was followed for 60 min using a fixed concentration of $DPPH^{\bullet}$ (71 µM) and a fixed concentration of selenohydantoin derivatives corresponding to the EC_{50} determined with the DPPH kinetic mode. For this part, we only kept GSH as the control because ebselen did not show any $DPPH^{\bullet}$ reduction capacity and our sulfur

and selenium derivatives share more common behavior with GSH than with ascorbic acid. Subsequently, we selected the most promising selenohydantoin derivatives based on their EC_{50} values for further evaluation. We graphically determined the time required for selenohydantoin derivatives at the EC_{50} to reach reaction completion (TEC_{50}). Indeed, the faster the reaction reaches completion, the more efficient the antioxidant activity is.[75] Then, the antiradical efficiency (AE in min^{-1} μM^{-1}), which is a kinetic-based parameter that combines reaction velocity (TEC_{50} in min) and antioxidant efficiency (EC_{50} in µM) was calculated for each selenohydantoin derivative as follows:

$$AE = \frac{1}{TEC_{50} x \, EC_{50}}$$

DPPH$^{\bullet}$ reduction is a *pseudo*-first-order kinetic reaction since it isinfluenced by both reactants and their concentrations.[75] So, k_1, the rate constant of the reaction was obtained from the slope of the non-linear *pseudo*-first-order equation ($y = a\, e^{-bx}$) where b represents k_1. Moreover, the k_1 value was also affected by the concentration and specific properties of the compounds tested. Thus, the rate of DPPH$^{\bullet}$ reduction could be better defined by k_2 calculated using the stoichiometric coefficient determined in the DPPH$^{\bullet}$ akinetic mode[76] as follows:

$$k_2 = \frac{k_1}{Stoichiometry\ coefficient}$$

3.4. Ferric Reducing Antioxidant Power (FRAP)

The Ferric Reducing Antioxidant Power (FRAP) assay is a non-radical antioxidant assay standing for the ability of antioxidant molecules to reduce ferric ions (Fe^{3+}). Indeed, the reduction of a Fe(III)-TPTZ complex to a Fe(II)-TPTZ by an antioxidant induces a change in absorbance at 593 nm with the appearance of an intense blue color which is proportional to the reducing power of the antioxidant. The FRAP reagent was prepared as follows: 100 mL aceto-acetic buffer (0.3 M, pH 3.6) was mixed with 10 mL of 2,4,6-*tris*(1-pyridyl)-5-triazine (TPTZ) (0.01 M solution in HCl 40 mM) and 10 mL of $FeCl_3$ (0.02 M in aceto-acetic buffer). The FRAP reagent was heated for 5 min at 37 ± 2 °C before use. Then, 0.1 mM to 1 mM of selenohydantoin derivatives were mixed with the FRAP reagent. After 5 min of incubation at 37 ± 2°C, the absorbance was read at 593 nm on a Shimadzu UV-VIS spectrophotometer (UV-2600). Calibration curves were carried out in a range of 0.1 mM to 1 mM with control molecules such as

ebselen, ascorbic acid, reduced glutathione, and $FeSO_4 \cdot 7H_2O$. The FRAP value was calculated for each molecule as follows:[46,47]

$$FRAP\ Value\ (\mu M) = \frac{Abs_{593\ nm}\ Antioxidant\ at\ 1000\ \mu M}{Abs_{593\ nm}\ \mathrm{FeSO_4.7H_2O}\ \ at\ 1000\ \mu M} \times 1000\ \mu M$$

3.5. Glutathione Peroxidase-like activity

GPx catalyzes the reduction of numerous hydroperoxides (organic hydroperoxide, H_2O_2, and lipid peroxides) with the concurrent oxidation of GSH. The formation of oxidized glutathione (GSSG) is quantified indirectly by following the consumption of nicotinamide adenine dinucleotide phosphate (NADPH) by glutathione reductase (GR), which reduces GSSG to GSH. Concentrations of *tert*-butyl hydroperoxide (^{t}BuOOH), cumene hydroperoxide, and H_2O_2 were 2 mM, 1.5 mM, and 1 mM, respectively. The concentration of GR was 1 U mL^{-1}, and GSH was used at 1 mM. The decrease in NADPH (0.15 mM initial concentration) absorbance was followed at 340 nm using a Shimadzu UV-VIS spectrophotometer (UV-2600) for 5 min for H_2O_2 or 30 min in the case of ^{t}BuOOH or cumene hydroperoxide as the reaction is slower. Solutions were prepared in potassium phosphate buffer 100 mM with 1 mM EDTA, pH 7.0 in ultrapure water (18.2 MΩ cm). Meanwhile, stock solutions of ebselen and selenohydantoin derivatives were prepared at 2 mM in DMSO and diluted to 20 µM (working concentration) in potassium phosphate buffer. The GPx-like activity was calculated as the slope of the linear part of the reaction ($\Delta Abs_{340\ nm}\ s^{-1}$) divided by ε_{NADPH}.

3.6. Computational calculations

Calculations were carried out using the Gaussian 09 package (revision D.01).[77] The analysis was carried out at 298.15 K and 310.15 K using a pressure of 1.01 bar. The Density Functional Theory (DFT) was used in the determination of all structures, molecular complexes, and transition states, using the hybrid M06-2X functional and the 6-311G (d, p) basis set (grid=ultrafine). Geometries were optimized using the solvation model based on density (SMD) for water, methanol, or DMSO. The vibrational analysis of each structure was performed to confirm the identity of all stationary points and was used in the attainment of thermal corrections to the enthalpy and the Gibbs free energy. Transition states were optimized using the Berny

algorithm and presented a single imaginary frequency (the intrinsic reaction coordinate (IRC) analysis was also carried out).

For the determination of the Gibbs distribution, the following equation was employed:

$$K = e^{-\Delta G/RT} = \frac{\%_B}{\%_A}$$

with R = 0.001987 kcal mol^{-1} K^{-1} and T = 298.15 or 310.15 K.A and B are the possible species involved in the equilibrium.

3.7. Cell culture and cytocompatibility

Primary Human Aortic Smooth Muscle Cells (HuAoSMC, CC2571) from Lonza were cultivated in a 75 cm^2 flask at 37 °C in a humidified incubator (10% CO_2). The culture medium (Clonetics™ SmGM™-2 BulletKit™, CC-3182) was composed of smooth muscle cell basal medium (SmBM™) added with 5% (v/v) of fetal bovine serum, 0.1% (v/v) of insulin, gentamicin sulfate amphotericin, human epidermal growth factor, and 0.2% (v/v) of human fibroblast growth factor B. To evaluate the cytocompatibility of selenohydantoin derivatives, HuAoSMC were seeded in 96-well plates at 60 x 10^{-3} cells/well in a complete medium. Selenohydantoin derivatives from 1 µM to 100 µM in potassium phosphate buffer/0.01% DMSO were incubated with HuAoSMC for 24 h. Then, MTT reagent at 5 mg mL^{-1} was added in each well (without removing the medium), and incubated for 3 h at 37 °C (in 10% CO_2). Then, purple formazan crystals were dissolved with 50 µL of DMSO and the absorbance was read at 570 nm with a reference at 630 nm using an EL 800-microplate reader (Bio-TEK Instrument, France).[78] The metabolic activity in the presence of selenohydantoin derivatives was compared to the control condition, *i.e.* culture medium with 0.01% DMSO considered as 100% viability.

3.8. Statistical analysis

Assays were performed in triplicate of three different experiments (n=3), results were presented as means ± standard error of the mean and were compared using One-way ANOVA, and Bonferroni post-test (GraphPad Prism version 5 software); $p < 0.05$ was considered as statistically significant.

4. Conclusions

A series of new selenohydantoin derivatives has been synthesized employing relatively straightforward protocols, beginning with commercially available starting materials, mild conditions, and good yields. Unlike ebselen, each of the selenohydantoin derivatives showed general antioxidant properties regarding DPPH and FRAP. In particular, compound **5e** with trifluoromethyl substitution displayed a GPx-like activity that is comparable to ebselen. Notably, our compounds exhibit greater hydrophilicity and cytocompatibility compared to ebselen, making them attractive candidates for drug development. The presence of selenone/selenolate forms, as determined by DFT calculations, may contribute to the stability and activity of these compounds. Further studies are needed to confirm the GPx-like activity of our selenohydantoin derivatives and their potential use as an antioxidant defence against oxidative stress.

Declaration of Competing Interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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2.2 Conclusions

Selenoneine was discovered around a decade ago, and since then, it has been studied and evaluated. Many efforts have been placed into its synthesis and isolation. However, this has been proven to be a rather complicated, and lengthy process, and might carry environmental and economic burdens. Thereafter, our goal was to overcome these obstacles and to obtain active molecules with resemblance to selenoneine. Thus, a series of novel selenohydantoin derivatives has been synthesized successfully using straightforward procedures, starting from readily available materials, under mild reaction conditions, and yielding good quantities of the desired products. In contrast to ebselen, each of these selenohydantoin derivatives has demonstrated promising general antioxidant properties when assessed through DPPH (akinetic and kinetic modes) and FRAP assays and compared to standard controls, such as ascorbic acid, glutathione, and ebselen as a selenium-containing molecule.

Furthermore, the selenohydantoins were investigated as GPx mimics. Their potential to reduce organic or inorganic peroxides was evaluated and compared to ebselen and selenocystamine. Indeed, **5d**, **5e,** and **5f** exhibited the best activity among the selenohydantoins studied, especially in reducing hydrogen peroxide. Of particular interest is **5e**, featuring a trifluoromethyl substitution, which exhibited a remarkable GPx-like activity comparable to that of ebselen. In addition, the presence of selenone/selenolate forms in these selenohydantoins, as shown through DFT calculations, suggests their contribution to both stability and activity

Notably, the new selenohydantoins possess enhanced hydrophilicity and cytocompatibility compared to ebselen and selenocystamine, rendering them compelling candidates for potential drug development. Further comprehensive investigations are needed to explore their potential applications to oxidative stress-related conditions, especially in the frame of cardiovascular illnesses, as well as their interaction with the essential cardiac components, such as the *S*-nitrosothiols, hence enhancing nitric oxide bioavailability and bioactivity.

3 Chapter III: Selenohydantoins and nitric oxide bioavailability

3.1 Nitric oxide release studies

In this chapter, we will discuss the ability of the new selenohydantoins with GPx-like activity to promote the release of a bioavailable •NO from RSNO. The bioavailability of •NO will be verified upon •NO transfer into muscle cells to promote the formation of a •NO pool mobilizable to induce vasorelaxation of rat aortic rings.

To select the most potent selenohydantoins to release •NO, we first checked *in tubo* the ability of selenohydantoins to release •NO from three different RSNOs based on their origin, physiological such as GSNO (Table 1) and semi physiologic *S*-nitroso-N-acetylcysteine (NACNO) (Table 2) *versus* synthetic such as or *S*-nitroso-N-acetylpenicillamine (SNAP) (table 2), their physicochemical properties with higher hydrophilicity for GSNO than SNAP and their ability to release a bioavailable •NO with vasorelaxant properties [140,141].

Table 1: *Parameters of the nitric oxide (•NO) release activity of 50 µM Selenohydantoins (5 a-h) from 1 mM of* S-*nitrosoglutathione (GSNO), in the presence of 200 µM glutathione (GSH) during one hour. Ebselen and Selenocystamine at 50 µM are used as control seleno-molecules. Results are presented as the mean ± SEM of three independent experiments and are compared using One-way ANOVA, and Bonferroni post-test, ** $p < 0.05$ *versus the selenocystamine. n/a: no activity.*

Nota Bene: *(Material and method are presented in the following article)*

	Parameters of •NO release activity		
Compounds	**Reaction speed (µM GSNO consumption/min)**	**Time to reach the end the reaction (min)**	**Remaining GSNO (µM) at the end of the reaction**
Control	n/a	n/a	n/a
Ebselen	n/a	n/a	n/a
Selenocystamine	33 ± 0	13.2 ± 0.2	595 ± 6
5a	20 ± 0*	27.1 ± 0.3	480 ± 5
5b	15 ± 4*	28 ± 1	579 ± 110
5c	28 ± 4	28.2 ± 0.7	210 ± 110*

5d	11 ± 4*	24.1 ± 0.4	738 ± 107*
➢ **5e**	74 ± 21*	12 ± 3	125 ± 98*
➢ **5f**	33 ± 11	26 ± 2	181 ± 237*
5g	15 ± 4*	21.2 ± 0.5	675 ± 84
5h	24 ± 4	26 ± 2	385 ± 91

Table 2: *Speed of the the nitric oxide (•NO) release activity of 50 µM Selenohydantoins (5 a-h) from 1 mM of* S-*nitroso-N-acetylcysteine (NACNO) or* S-*nitroso-N-acetylpenicillamine (SNAP) in the presence of 200 µM glutathione (GSH) during one hour. Ebselen and Selenocystamine at 50 µM are used as control selenomolecules. Results are presented as the mean ± SEM of three independent experiments and are compared using One-way ANOVA, and Bonferroni post-test, ** $p < 0.05$ *versus the selenocystamine. n/a: no activity*

	•NO release activity	
Compounds	**Reaction speed (µM SNAP consumption /min)**	**Reaction speed (µM NACNO consumption /min)**
Control	n/a	n/a
Ebselen	n/a	n/a
Selenocystamine	134 ± 0	67 ± 0
5a	7 ± 3*	n/a
5b	6 ± 0*	n/a
5c	7 ± 3*	36 ± 8*
5d	11 ± 4*	n/a
5e	11 ± 0*	38 ± 4*
5f	7 ± 3*	n/a
5g	7 ± 3*	11 ± 4*
5h	7 ± 3*	71 ± 8

Interestingly the molecules showed different activities within themselves and upon the different RSNOs. Indeed, •NO release activities of selenohydantoins are much more efficient with GSNO, especially for **5e** that exhibited a significant activity to decompose GSNO that was even higher than selenocystamine, whilst **5f** was comparable to selenocystamine. Furthermore, selenohydantoins were quite inefficient to decompose SNAP, unlike selenocystamine that was the most active. In addition, only **5h** was able to decompose NACNO as efficiently as selenocystamine. On another note, ebselen did not show any catalytic activity and was not able to decompose any of the RSNOs studied under those conditions. These activities suggest more complex mechanistic pathways, which involve the

interaction between the structures of RSNOs and selenohydantoins, and which could also depend on the choice of the thiol used, where GSH could work better with its nitrosated form the GSNO. additionally, the NACNO and SNAP might have an inhibitory effect on molecules with GPx-like and catalytic activities [23,26,30–32]. Indeed, numerous studies explored the interaction between SNAP and GPx. It was demonstrated that SNAP inhibit the GPx, probably *via* modification and oxidation of essential residues such as cysteine (Cys) and selenocysteine (Secys). This phenomenon may explain the observed lack of activity with SNAP in our *in tubo* experiment [41–44].

Furthermore, as GSNO is the physiological •NO store and **5e** and **5f** exhibited the highest GPx-like activity as well as the highest activity to release •NO from GSNO *in tubo*, we decided to go further *in vitro* and *ex vivo* with these three molecules. Therefore, the ability of **5e** and **5f** to induce the release of •NO and thus promoting the formation of a •NO pool in human smooth muscle cells will be evaluated *in vitro* through the quantification of intracellular nitrite ions and protein *S*-nitrosation. The impact of **5e** and **5f** on cellular GPx activity as well as on intracellular GSH concentrations will be assessed. Finally, the capacity of **5e** and **5f** to mobilize the •NO store, thus promoting *ex vivo* vasorelaxation of isolated rat aortic rings will be demonstrated. All these investigations will be presented on the form of a manuscript (to be submitted to Biochemical Pharmacology).

Article V: **Selenohydantoins Improving Vascular Homeostasis by modulating Nitric Oxide Bioavailability and Vasorelaxation**

Rama Alhasan, Caroline Perrin-Sarrado, Isabelle Fries-Raeth, Claus Jacob, Caroline Gaucher

(To be submitted)

Abstract

The intricate regulation of vascular homeostasis plays a pivotal role in maintaining cardiovascular health. This study delves into the potential modulation of vascular function through the introduction of new selenohydantoins, a class of molecules with emerging pharmacological significance. The objective was to assess their effects on nitric oxide (•NO) release from *S*-nitrosoglutathione (GSNO) and •NO bioavailability for smooth muscle cells, and subsequent vasorelaxation activity on aortic rings. The investigation employed a comprehensive methodology to analyze the interaction between two interesting selenohydantoins bearing glutathione peroxidase (GPx)-like activity such as **5e** with a trifluoromethyl (CF_3) substitution and **5f** with a methyl (CH_3) substitution, and *S*-nitrosoglutathione (GSNO). First, we demonstrated *in tubo* that **5e** and **5f** were able to release •NO from GSNO with a higher efficiency than selenocystamine in the case of **5e**. This finding was confirmed *in vitro* where **5e** co-incubation with GSNO raised significantly the formation of an intracellular •NO pool without affecting cell redox balance (GPx activity and intracellular GSH concentration), materializing •NO bioavailability and potential positive cardiovascular effect. Indeed, **5e** was also shown to be efficient *ex vivo* to mobilize GSNO-induced •NO pool in aorta for vasorelaxation.

This study sheds a light on promising therapeutic applications of these selenohydantoins, especially for conditions characterized by impaired endothelial function and opens avenues for further exploration into the broader physiological and pathological applications.

Selenohydantoins Improving Vascular Homeostasis by modulating Nitric Oxide Bioavailability and Vasorelaxation

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Abstract

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effect. Indeed, **5e** was also shown to be efficient *ex vivo* to mobilize GSNO-induced •NO pool in aorta for vasorelaxation.

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Keywords: Selenohydantoins, Redox, Nitric oxide, *S*-nitrosothiol, Human Aortic Smooth Muscle, Endothelium, Aorta.

1. Introduction

The connection between *S*-nitrosothiols (RSNO), nitric oxide (•NO), its release and bioavailability, *S*-nitrosation process, and redox modulation constitutes a complex and dynamic network of molecular interactions that profoundly influences cell signaling, redox homeostasis, and the pathophysiology of various health conditions, especially in the context of cardiovascular diseases. This multifaceted interrelationship has garnered significant attention in biomedical research.

In the endothelium, •NO is synthesized by nitric oxide synthase (eNOS), from L-arginine, and BH_4 as a cofactor [1]. •NO can diffuse through cell plasma membranes in the vascular wall, causing smooth muscle cell relaxation by a cGMP-dependent mechanism regulating vascular tone. Endothelial-derived •NO can also diffuse into the vessel lumen, where it prevents platelet aggregation and adhesion to the endothelium also by a cGMP-dependent mechanism [2–6]. Therefore, endothelial dysfunction represents the major pathophysiological link between exposure to cardiovascular risk factors and the development of atherosclerotic disease. It manifests as the earliest stage of vascular diseases with the development of plaques followed by their rupture and intravascular thrombosis [7,8]. Endothelial dysfunction is commonly attributed to a reduction in •NO production, resulting in the endothelium inability to initiate vasodilation in response to vasodilatory stimuli such as acetylcholine or shear stress. The reduced •NO production is linked to eNOS uncoupling resulting in the production of $O_2^{\bullet-}$ instead of •NO. Then, $O_2^{\bullet-}$ reacts with •NO producing peroxynitrite ion ($ONOO^-$), a highly reactive oxidant that participates in the decrease of •NO bioavailability. eNOS uncoupling is frequently initiated in oxidative environments when BH_4 levels are low, either due to a reduction in its synthesis or an increase in its oxidation. This condition marks an initial and reversible phase in the progression of atherogenesis. Consequently, the early clinical

identification of endothelial dysfunction may emerge as a critical tool in preventing or reversing the progression toward atherosclerosis and ischemic heart disease [7–9].

Furthermore, *S*-nitrosation, a reversible posttranslational modification of proteins, involves the binding of •NO to cysteine residues and plays an important role in the cardiovascular modulation of various proteins and a crucial role in protecting •NO and proteins against oxidative stress. •NO selectively *S*-nitrosates endothelial proteins, influencing processes like migration, permeability, oxidative stress, aging, and inflammation. Additionally, nitrite ions (NO_2^-) serve as a precursor for the generation of bioactive •NO adducts such as *S*-nitrosothiols (RSNOs) when interacting with reduced thiol functions, and Dinitrosyl Iron Complexes (DNIC) when interacting with ferrous ion (FeII) [10,11]. These adducts have been proposed as significant intracellular •NO reservoirs, which may function as a key player in •NO bioactivity [12,13]. Recent studies suggest the complexity of protein *S*-nitrosation status *in vivo*, involving a precisely regulated equilibrium between *S*-nitrosation and denitrosation reactions [14–16]. RSNOs derived from proteins, peptides, and amino acids maintain intracellular and •NO bioactivity, playing key roles in human health and disease [17]. RSNOs decompose spontaneously and slowly release •NO, a reaction that can be catalysed by many factors [18]. Moreover, *S*-nitrosation of proteins present in the blood stream mediates •NO bioactivity and diffusibility from its initial site of synthesis that ranges around 100-200 μm, with diffusion constant of 3300 $\mu m^2/s$, thus, helping to improve its half-life and radius of action [19]. Indeed, RSNOs play a role in either promoting the release of •NO either spontaneously or catalytically[20,21], or forming a store of •NO within vascular smooth muscle cells. Afterwards, •NO can diffuse freely in plasma membranes and enter in smooth muscle cells or platelets to activate soluble guanylate cyclase (sGC). Alternatively, RSNOs can *S*-transnitrosate thiol groups on the cell membrane surface, initiating a cross-membrane signalling series of events that result in the transport of the •NO moiety into the cell [22,23]. RSNOs can also cross the cell membrane by converting into either I) *S*-nitroso-L-

cysteine (L-cysNO), which can be transported into the cell *via* the L-type amino acid transporter or II) L-cysNO-glycine, only formed by the hydrolysis of GSNO *via* γ-glutamyl transpeptidase before being taken into the cell by dipeptide transporters [24–27].

The reaction of •NO with low molecular weight thiols like glutathione (GSH) results in *S*-nitrosoglutathione (GSNO), which is the main storage form of •NO within cells and the main physiologic mediator of •NO-dependent signal transduction in cells [28]. In addition, there has been a huge interest in GSNO as a potential therapeutic agent [29]. Understanding the mechanisms underlying •NO release from GSNO and its impact on protein *S*-nitrosation is essential for unraveling the complex relationship between •NO signaling, oxidative stress, and redox modulation. The presence of intracellular •NO storage forms has captured the interest of researchers for more than three decades. Notably, relaxation responses were observed in response to light even in endothelium-removed vessels [30,31]. Moreover, our lab achieved the *in vivo* formation of a •NO store in rat aorta by oral administration of GSNO-loaded nanocomposites [32]. Thereafter, our lab proved that this •NO store can be mobilized for rat aorta vasorelaxation and extended this *ex vivo* observation to other RSNOs [32,33]. The bioavailability of the •NO vascular store was evaluated with *N*-acetylcysteine (NAC) that is able to enter inside the cells and displaces •NO from RSNOs by transnitrosation process, inducing vasorelaxation of precontracted rat aortic rings. On the contrary, in the absence of •NO store, NAC failed to produce aortic ring vasorelaxation. This study also proposed a potential treatment that could improve endothelial function using endothelium-removed aortic rings in direct comparison with endothelium-intact aortic rings [33,34].

Moreover, in biological systems, •NO is primarily oxidized to form nitrite ions, a process that renders it stable for several hours. Subsequently, these nitrite ions can further oxidize to become nitrate ions (NO_3^-) [35]. Within the cell cytoplasm, an environment characterized by its reductive properties, the predominant oxidized form of •NO is in the shape of nitrite ions. In the

cardiovascular system, •NO can produce remote or long-lasting effects by the formation of various RSNOs, thus ensuring slower but much more persistent effects than does free •NO [28]. Moreover, under physiologic oxidative stress, •NO might protect cells of some censorious protein thiols by *S*-nitrosation, preventing them from further oxidative damage. GSNO can modify protein thiols *via* *S*-nitrosation or glutathionylation, which strictly depends on the surrounding redox equilibrium. •NO can be released from RSNO by enzymes such as gamma-glutamyltransferase specific for GSNO or redoxins like protein disulfide isomerase or the thioredoxin system to achieve its physiological activities. Denitrosation can also be achieved by GSNO reductase [36]. Organoselenium molecules have been discussed to possess intriguing catalytic properties that could also influence •NO biology in significant ways. They serve a dual role by enhancing the bioavailability of •NO and facilitating protein *S*-nitrosation. These processes are pivotal in the context of cardiovascular diseases and overall health [37,38].

Selenium is an essential trace element that compose various enzymes and proteins, known as selenoproteins, which have antioxidant properties and help protect cells from oxidative damage caused by peroxides and free radicals. One eminent member of selenoproteins is the glutathione peroxidase (GPx) family, which is involved in protecting cells from oxidative damage [39–41]. In addition, sufficient selenium intake has also been proven to be critical to proper physiological function and decrease the risk of cardiovascular diseases [42]. Interestingly, organoselenium molecules has emerged as a significant area of interest in modulating •NO availability and redox signaling pathways with implications for cellular function and health [43,44].

Indeed, organoselenium molecules have been shown to catalytically generate •NO from RSNO in the presence of appropriate thiol-reducing agents, offering potential applications in sustained •NO release for biomedical purposes [44]. In addition, an enhancement in *S*-nitrosated proteins within endothelial cells occurs in the presence of selenium derivatives especially diselenide (*e.g.* selenocystamine), and GSH as a reducing agent, along with GSNO as •NO donor [45–49].

Studies have demonstrated the use of selenium-containing polyurethane with elevated catalytic stability for sustained •NO release from GSNO, highlighting the potential for practical applications in biomedical devices for long-term blood-contacting applications [50]. Additionally, organoselenium-derivatized polymers such as organoselenium modified polyethyleneimine have been shown to effectively generate •NO from GSNO. Moreover, their immobilization in layer-by-layer assembly of sodium alginate and organoselenium modified polyethyleneimine on quartz and polymeric substrates has been explored as a method to create •NO-generating surfaces [44]. These studies have paved the way for the employment of organoselenium molecules to ensure the sustained and controlled •NO delivery from RSNOs, with potential implications for biomedical applications such as cardiovascular therapeutics, wound healing, and antimicrobial treatments. GSH presence is essential as a reducing agent, which produces a selenosulfide and finally selenol/selenolate ($RSeH/RSe^{-} + H^{+}$), key player species in RSNO decomposition [51,52]. Recent studies have discussed the possible involvement of SeNO species in •NO release, where an intermediate of RSeNOs can form via transnitrosation from RSNOs to selenols, which then rapidly form the diselenide with stimioulinous release of •NO.

We have previously synthesized a series of selenohydantoins that were inspired by selenoneine [53]. These selenohydantoins exhibited general antioxidant activities and GPx-like catalytic activity and were cytocompatible with human aortic smooth muscle cells (HuAoSMC) even at 100 μM [53]. Moreover, density functional theory calculations demonstrated that the selenolate/selenone forms are viable in solution, which is probably contributing to the activities. The molecules exhibiting the most robust activity in this regard were selected for further *in vitro* analysis.

The primary objective of this analysis is to investigate whether these selenohydantoins can effectively increase the bioavailability of •NO within HuAoSMC. The ability to modulate •NO levels, make them intriguing candidates for potential therapeutic applications and further

investigation within the cardiovascular system as well as their ability to affect the cellular GPx-activity and GSH levels in HuAoSMC. Furthermore, because •NO release and bioavailability carry significant importance in vascular research, the interesting selenohydantoins will be studied *ex vivo* employing isolated rat aortic rings. These studies present a tool for investigating vascular function and the critical role of •NO. Isolated rat aortic rings offer a physiologically relevant model to assess endothelial function. They also enable the examination of endothelium-dependent and independent vasodilation under different conditions. On one hand, it is feasible to explore the response of aortic rings to •NO donors such as the RSNOs or substances that modulate •NO vascular store and release. On the other hand, it helps to assess the vasodilatory effects of potential therapeutic agents aimed at regulating the redox balance, which holds relevance for conditions like hypertension and endothelial dysfunction. Moreover, agents such as organoselenium molecules are considered as potential targets in the treatments of CVDs and have been under research for decades. Therefore, our objective was to evaluate if the new selenohydantoins could hold vasoactivities and could enhance •NO bioavailability.

2. Material and methods

2.1. Chemicals

Ebselen and selenocystamine were purchased from Sigma-Aldrich, dimethyl sulfoxide (DMSO) and phosphate buffer solution were used respectively as solvents. All commercially available reagents were purchased from Sigma-Aldrich or Alfa Aesar and used as received.

2.1.1 *S*-nitrosoglutathione synthesis

GSNO was synthesized and isolated as a powder in the lab according to previously published protocols and its purity was assessed by ultraviolet spectrophotometry using the specific molar absorbance of the S-NO bond at 334 nm (εGSNO = 922 M^{-1} cm^{-1}) [52] . All experiments and assays involving GSNO were performed with subdued lighting, to minimize light-induced degradation of RSNOs.

2.1.2 Selenohydantoin synthesis

The synthesis protocol involves the preparation of 5-aryl-2-selenoxoimidazolidin-4-ones derivatives (*i.e.* **5e** and **5f**) through a series of reactions that has been already detailed in our prior research article [54], and summarized procedure as follows:

a) Preparation of (Z)-5-arylidene-2-thioxoimidazolidin-4-ones:

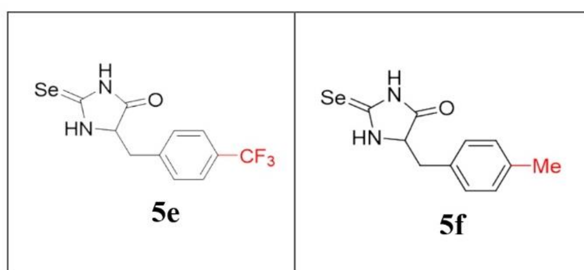
Combination of thiohydantoin and aromatic aldehyde in water with piperidine. Followed by Heating, filtration, washing, and drying the resulting precipitate.

b) Synthesis of (Z)-5-arylidene-2-(methylthio)-3,5-dihydro-4H-imidazol-4-ones:

Reaction of (Z)-5-arylidene-2-thioxoimidazolidin-4-one with acetonitrile, K_2CO_3, and iodomethane. Followed by Heating, then addition of water, filtration, washing, and purifying by silica gel chromatography.

c) Synthesis of 5-aryl-2-selenoxoimidazolidin-4-ones (**5e** and **5f**):

Starting with reacting Se° in ethanol with $NaBH_4$ to obtain a colourless solution. Followed by the addition of the respective (Z)-5-arylidene-2-(methylthio)-3,5-dihydro-4H-imidazol-4-one at a room temperature. Then, Heating, filtration through silica gel, drying over $MgSO_4$, and finally isolate the final product. The structures were confirmed using various spectroscopic techniques, including ^{1}H NMR, ^{13}C NMR, ^{77}Se NMR, and HRMS.



2.2. *In tubo RedoX catalytic release of nitric oxide*

We investigated the nitric oxide release activity of selenohydantoins *in tubo,* utilizing GSNO as a •NO donor, and GSH as a reducing agent. Initially, 1 mM of GSNO solution

extemporaneously prepared in a phosphate buffer solution pH = 7.4 was mixed with 200 µM of GSH [48,55,56]. Then, 50 µM of **5e** and **5f** were added to start the catalytic release of •NO from GSNO. GSNO decomposition was followed spectrophotometrically at 334 nm wavelength in quartz cuvette over one-hour time which also corresponds to the end of activity and the stabilization of GSNO degradation.

2.3. *In vitro RedoX catalytic release of nitric oxide*

2.3.1. *In vitro* Human Aortic Smooth Muscle cell model and incubation conditions

Primary Human Aortic Smooth Muscle Cells (HuAoSMC, CC2571) from Lonza were cultivated in a 75 cm^2 flask at 37 °C in a humidified incubator (10% CO_2). The culture medium (Clonetics™ SmGM™-2 BulletKit™, CC-3182) was composed of smooth muscle cell basal medium (SmBM™) added with 5% (v/v) of fetal bovine serum, 0.1% (v/v) of insulin, gentamicin sulphate amphotericin, human epidermal growth factor, and 0.2% (v/v) of human fibroblast growth factor B. HuAoSMC were seeded at 60.10^3 cells/mL in a complete medium in a six wells plate for *in vitro* evaluations. For all experiments, cells were seeded in a six well plate at 3500 cells/cm^2 two days before incubation.

To define the suitable GSNO, and **5e** and **5f** concentration, HuAoSMC were first incubated with 1 µM to 100 µM of GSNO alone or with 1 µM to 100 µM of **5e** and **5f** for one hour to investigate their self-capacity to modulate intracellular nitrite ions or RSNO concentration. Mixing those results with the **5e** or **5f** over GSNO ratio used *in tubo* assay, we set **5e** and **5f** at 2.5 µM to monitor their catalytic capacity to release •NO from 50 µM GSNO either in co-incubation or sequential incubation mode.

Two different sets of HuAoSMC incubation with **5e** or **5f** and GSNO were designed. HuAoSMCs were either co-incubated with 2.5 µM **5e** or **5f**, and 50 µM GSNO during one hour

or sequentially incubated thirty minutes with 50 µM GSNO followed by the addition of 2.5 µM **5e** or **5f** for another thirty minutes or *vice versa*.

2.3.2. Nitric oxide bioavailability for smooth muscle cells

The bioavailability of •NO for HuAoSMC was followed by quantifying intracellular nitrite ions and RSNO concentration. Briefly, after co-incubation or sequential incubation of HuAoSMCs with **5e** or **5f** and GSNO, cells were washed and lysed in 50 mM Tris buffer pH 6.8 added with 150 mM NaCl, 1% (v/v) Igepal CA-630, 0.1% (v/v) SDS, 1 mM EDTA, 0.1mM neocuproine, 20 mM sodium tetraborate and 10 mM NEM. DAN or DAN, Hg^{2+} were added.

Nitrite ions and RSNO are quantified using a fluorometric based on the direct reaction between the non-fluorescent aromatic diamino compound 2,3-diaminonaphtalene (DAN) and nitrite ions to yield 2,3-naphtotriazole (NAT) a highly fluorescent product (DAN assay). For RSNO quantification, mercuric ions are used to cleave the S-NO bond allowing the release of nitrite ions that are quantified with DAN (DAN, Hg^{2+} assay). The fluorescent intensity is read on 100 µL of sample at 375 nm (λex = 375 nm/λem = 415 nm; JASCO FP-8300, France) [35]. A standard curve of 0.1 µM to 2 µM of nitrite ions or GSNO was built in parallel. The RSNO concentration (DAN, Hg^{2+} assay) is obtained by the subtraction of the total nitrite ions concentration (DAN assay). Results are expressed as nmol of nitrite ions or RSNO per mg of proteins (quantification of proteins see material and methods).

2.3.3. Redox impact on intracellular reduced glutathione concentration

As the release of •NO from GSNO using **5e** and **5f** might be based on a catalytic cycle consuming GSH, we monitored intracellular GSH concentration using 2,3-naphthalene dicarboxaldehyde as previously described [57]. After, co-incubation or sequential incubation of HuAoSMCs with **5e** or **5f** and GSNO, cells were lysed with cold 3.3% (v/v) perchloric acid and centrifuged at 10,000 *g* for fifteen minutes at +4 °C. After centrifugation, the supernatant from each tube was neutralized with 40% (v/v) NaOH and diluted at 1/5 in 0.1 M HCl containing 2

mM of EDTA. The pellet was used to quantify proteins. Diluted supernatants were deposited in triplicate of 60 µL in a black 96-well plate and added with 120 µL of 0.4 M borate buffer pH 9.2 and 20 µL of 5.4 mM 2,3-naphthalene dicarboxaldehyde, which specifically react with GSH. The plate was incubated for 2twenty five minutes, on ice, and in the dark before the reading GSH-NDA adduct fluorescence intensity at F528/20 nm after excitation at F485/20 nm with a spectrofluorometric instrument JASCO FP-8300, France. Intracellular GSH concentration is calculated upon a GSH calibration curve in the range of 0.65 µM to 3.25 µM. The values are expressed as nmol of GSH per µg of proteins.

2.3.4. Glutathione peroxidase activity *in vitro*

HuAoSMC co-incubated or sequentially incubated with **5e** or **5f** and GSNO, they were rinsed then lysed with Tris-HCl buffer (10 mM; pH = 7.8) and scraped. Later on, they were incubated for fifteen minutes at 4°C and followed with five thermal shocks using liquid nitrogen. Samples were then homogenized using a "shaker". The GPx activity of cells was investigated utilizing the lysate. Potassium-phosphate buffer 0.1 M, pH 7.0, 1 mM EDTA, 1 mM NaN_3, 0.2 mM NADPH, GR 2 U/ ml, 1 mM GSH, 100 µl of the sample (or H_2O for the blank) were all added in a cuvette then mixed by inverting then incubated for five minutes a 37 °C. After five minutes, 100 µL of 1.5 mM CHP or H_2O_2 were added and the absorbance was read at 340 nm by recording the trend for five minutes. The values are expressed as units per g of proteins of the mean of three independent experiments done in triplicates ± SEM.

2.3.5. Protein quantification assay by bicinchoninic acid (BCA) method

Proteins were quantified using the BCA Protein AssayTM (Ref: PierceTM BCA Protein Assay Kit). A 25 µL volume of cell lysate or cell pellet (GSH experiment) resuspended in 100 µL of 0.1M NaOH, then vortexed and deposited in 96-well plate and added with 200 µL of working reagent (mixture of copper and bicinchoninic acid) (kit BCA Pierce). After thirty minutes of incubation at 37°C and the absorbance was read at 570 nm with the plate reader (Ref: EL-800

BioTek). Protein concentration was calculated upon a BSA calibration curve ranging from (25-1000 µg / mL) (m/v) prepared in the cell lysis buffer or pellet resuspension buffer. Protein concentration was use to normalize all cell experiments.

2.4. Ex vivo nitric oxide bioavailability for aorta vasorelaxation

2.4.1. Animal and ethical statement

The experiments were conducted on 4- to 5-month-old male normotensive outbred Wistar rats (HanRj:WI, Janvier, Le Genest-Saint-Isles, France, RGD Cat# 13792727, RRID: RGD_13792727), a commonly used physiological model. Animals were housed in the "ACBS" animal facility (agreement number C54-547-30), in a rodent-specific pathogen-free area. Environmental variables such as temperature, humidity, ventilation, pressure gradient and light exposure are controlled independently for each room. Rats ate standard rat chow (A04, Safe, Villemoison-Epinay sur Orge, France) and drank water (Aqua-clear®, Culligan, Nortbrook, USA) *ad libitum*. All experiments were performed in accordance with the European Community guidelines (2010/63/EU) for the use of experimental animals in the respect of the 3Rs' requirements for Animal Welfare. The experimental protocols and procedures were approved by the regional ethics committee on animal experimentation (BisNitroVivo APAFIS referral #15598) and the out-of-field experimental procedures were controlled by the "ACBS" animal facility. Rats were anaesthetized with 4% isoflurane and sacrificed by decapitation. A segment of the descending thoracic aorta was removed and immediately placed in ice-cold Krebs' solution containing 119 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.6 mM $CaCl_2$, 24 mM $NaHCO_3$, 5.5 mM glucose, adjusted to pH 7.4. Vessels were cleaned from surrounding connective tissues, cut in 2-mm long rings and immediately used for vasoactivity studies. Vasoactivity was evaluated using an isometric tension recording system in 10 mL organ chambers (EMKABATH, Emka Technology, France).

2.4.2. *Ex vivo* vasorelaxant activities

Vasorelaxations of aortic rings were measured using an isometric tension recording system in 10 mL organ chambers (EMKABATH, Emka Technology, France), either on endothelium intact (E+) and removed aortic (E-) rings, where the endothelium was mechanically removed by gently rubbing the intimal surface of the vessel with forceps. For all experiments, the organ chambers were filled with 10 mL Krebs' solution continuously bubbled with O_2 at 37 °C. Following sixty-minutes equilibration at a basal resting tension of 2 g, rings were exposed 2 times to KCl (60 mM) to check viability. Endothelium integrity or removal was assessed using 10^{-5} M carbachol (CC) a muscarinic acetylcholine receptor agonist inducing vasorelaxation when the endothelium is intact, on aortic rings pre-contracted with 10^{-6} M PHE.

5e and **5f** were both evaluated for their capacity to induce vasorelaxation in two different conditions: 1) as a direct vasorelaxant effect of **5e** and **5f,** 2) as the "indirect effect" coming from the ability of **5e** and **5f** to catalytically release •NO (denitrosate) from nitrosated compounds such as RSNOs from the •NO store formed by preincubation of aortic ring with GSNO. **5e** and **5f** were solubilized in Milli-Q water and DMSO (final concentration of DMSO was 0.02 % (v/v) in the organ chambers). Therefore, all the experiments were performed in the presence of DMSO 0.02% (v/v). For direct vasorelaxant effect, concentration response curve was performed on aortic rings that were previously pre-contracted with 10^{-6} M PHE. For each molecule cumulative concentrations from 10^{-11} M to 10^{-5} M were added on endothelium intact and endothelium removed aortic rings (Fig.1).

Fig. 1. Protocol for concentration-response curves (CRC) of 5e or 5f on precontracted isolated rat aortic rings. K60: KCl 60 mM, PHE: Phenylephrine, CC: Carbachol

In another set of experiments, the ability of **5e** and **5f** to mobilize a •NO store formed in aorta by pre-incubation with GSNO was evaluated. In this model, GSNO induced the formation of a •NO store mainly composed of RSNO in the vessel wall [33]. Hence, we evaluated if **5e** and **5f** at 10^{-7}, 10^{-6}, and 10^{-5} M can catalytically release •NO from the vascular store and therefore induce vasorelaxation.

A •NO vascular store was created by incubating endothelium intact and endothelium removed aortic rings with 2 µM GSNO for thirty minutes, followed by three times washing (twenty minutes each) with Krebs' solution to eliminate excess GSNO. Then, pre-constricted aortic rings with 10^{-6} M PHE underwent different treatments to evaluate •NO store in the aortic wall either with 10^{-7}, 10^{-6} and 10^{-5} M of **5e** or **5f**, or with 10^{-5}, 10^{-4} and 10^{-3} M of N-acetylcysteine (NAC) as a positive control. Indeed, NAC alone did not show any vasorelaxant activities but was able to mobilize the •NO store created by pre-incubation with RSNO and therefore, vasodilation [33]. Furthermore, to ensure that the observed effects were due to •NO mobilization from vascular stores by **5e** or **5f** and not from their direct vasorelaxant effect, a control consisting of aortae not pre-incubated with GSNO was conducted (Fig. 2).

Fig. 2. Protocol of the vasorelaxant activity of 5e or 5f on isolated rat aortic rings pre-treated with GSNO then contracted with PHE. K60: KCl 60 mM, PHE: Phenylephrine, CC: Carbachol, GSNO: *S*-nitrosoglutathione.

2.5. Data and statistical analysis

All the results were presented as means ± standard error of the mean (SEM) and analysed (Graph Pad prism® software version 8.0.2); $p < 0.05$ was considered as significant.

Results of *in tubo* and *in vitro* quantifications were analyzed by the One-Way or Two-Way ANOVA test followed by a Bonferroni's multiple comparisons test.

Vasorelaxant responses of **5e** and **5f** were given as the percentage of relaxation from 10^{-6} M phenylephrine precontraction and calculated as in equation (1):

$$\% \, Vasorelaxation = \left(\frac{\Delta \, Tension_{5e \, or \, 5f}}{\Delta Tension \; of \; PHE}\right) x \; 100 \qquad (1)$$

ΔTension of **5e** or **5f** was calculated as (Tension of PHE 10^{-6} M, g - Tension **5e** or **5f**, g) and ΔTension PHE (Tension PHE 10^{-6} M, g -Tension baseline, g).

Each individual concentration response curve was investigated if it fits the Hill logistic equation (2) (Graph Pad prism® software version 8.0.2):

$$\% \, Vasorelaxation = E_{min} + \frac{(E_{max} - E_{min})}{(1 + 10^{((logEC50 - \log concentration) x \; Hill \, slope))})} \qquad (2)$$

For the second part of the *ex vivo* experiment, vasorelaxant effects were calculated using the equation (3), where **5e**, **5f** and NAC were evaluated for the ability to release •NO from the stores induced by preincubation with GSNO

$$\% \, Vasorelaxation = 100 - \left(\frac{\Delta \, Tension_{5e \, or \, 5f}}{\Delta \, Tension \; of \; PHE \; contraction}\right) x \; 100 \qquad (3)$$

Where ΔTension of **5e** or **5f** or NAC (was calculated as Tension **5e** or **5f or NAC**, g - Tension of baseline, g). ΔTension PHE contraction (value of the previous tension point recorded before adding the next **5e** or **5f** concentration, g - Tension baseline, g).

Analysis and comparisons of different treatments effects of **5e** and **5f** vasorelaxant effects, were performed by the One-Way or Two-Way ANOVA test followed by a Bonferroni's multiple comparisons test.

3. Results

3.1. in tubo RedoX catalytic release of nitric oxide

The catalytic release of •NO from GSNO by **5e** and **5f** selenohydantoins was evaluated in the presence of GSH as a reducing agent essential for GPx-like activity (Fig. C3 and D3). Indeed, in the absence of GSH, no catalytic release of •NO was observed (data not shown). In addition, GSNO alone or with GSH in the absence of **5e** or **5f** did not show any •NO release (Fig. A3 and B3). **5e** and **5f** were both able to catalyse the release of •NO and inducing an increase in absorbance at the onset of the reaction. whereas, ebselen a well-studied GPx mimic did not show any •NO catalytic release under the same conditions (Table 1). **5f** activity is two times slower than **5e** with a two times longer lasting activity while being as fast as selenocystamine with two times longer lasting

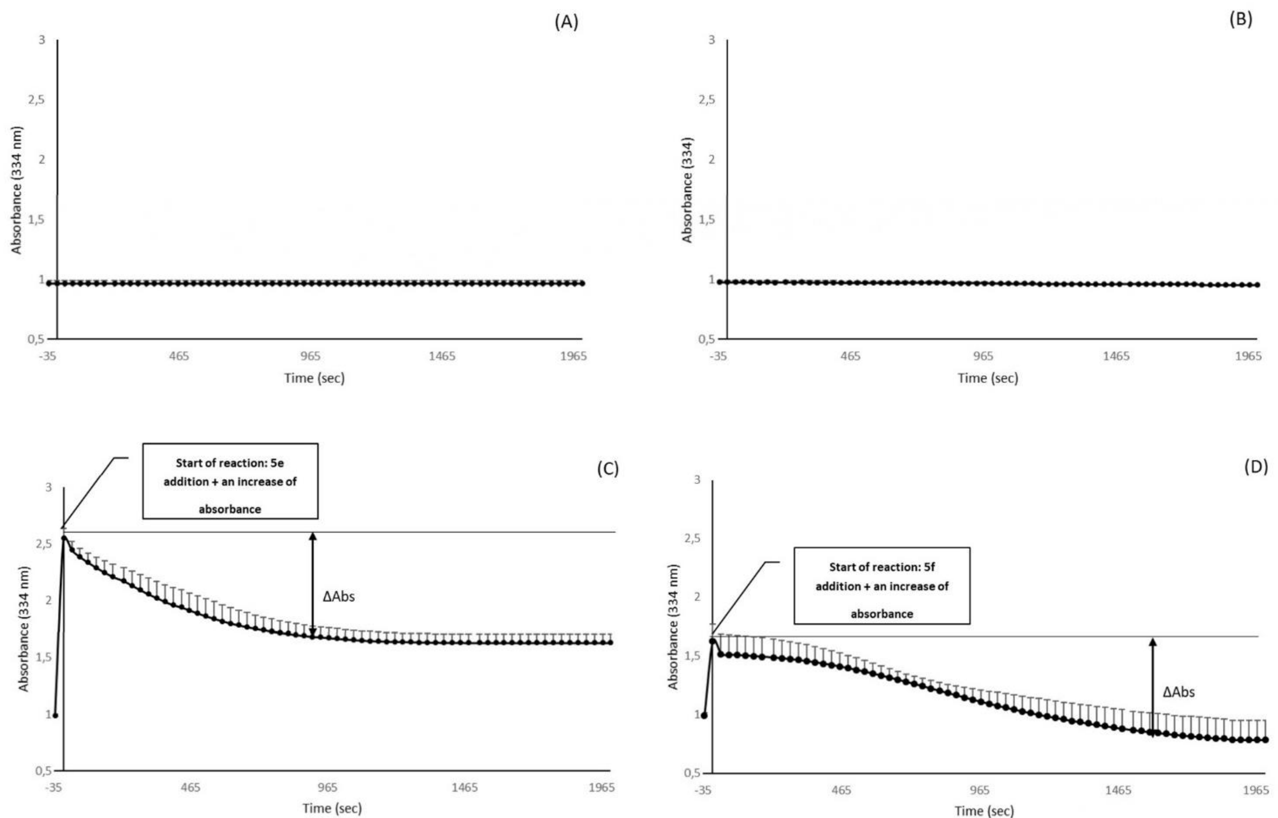


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Fig. 3. Nitric oxide (•NO) release activity from *S*-nitrosoglutathione (GSNO), (A) GSNO alone, (B) in the presence of glutathione (GSH), or (C) 5e and GSH or (D) 5f and GSH. Results are presented as the mean ± SEM of three independent experiments.

Table 1. Parameters of 5e or 5f nitric oxide (•NO) release activity from *S*-nitrosoglutathione (GSNO), in the presence of glutathione (GSH) during one hour. Ebselen and Selenocystamine are used as control seleno-molecules. Results are presented as the mean ± SEM of three independent experiments and are compared using One-way ANOVA, and Bonferroni post-test, * $p < 0.05$ *versus* selenocystamine. n/a: no activity

Compounds	Parameters of •NO release activity		
	Speed of the reaction(μM of GSNO consumption/min)	Time to reach the end of the reaction (min)	Remaining GSNO at the end of the reaction (μM)
Control	n/a	n/a	n/a
Ebselen	n/a	n/a	n/a
Selenocystamine	33 ± 0	13.2 ± 0.2	595 ± 6
5e	74 ± 21 *	12 ± 3	125 ± 98 *
5f	33 ± 11	26 ± 2	181 ± 237 *

As **5e** and **5f** were able to induce the release of •NO from GSNO along with their GPx-like activity *in tubo* (63 ± 1 and 55 ± 3 μM NADPH min^{-1}, respectively) [54], we then evaluated if the released •NO is bioavailable for human smooth muscle cells while preserving cell redox balance by stopping GSH overconsumption.

3.2. In vitro RedoX catalytic release of nitric oxide

3.2.1. Nitric oxide bioavailability for smooth muscle cells

•NO bioavailability for HuAoSMCs was evaluated through the intracellular quantification of nitrite ions, reflecting the oxidized version of free •NO in aqueous media, and RSNOs, a mix of *S*-nitrosothiols and *S*-nitrosoproteins. Both nitrite ions and RSNO forming the •NO pool in cells and tissues. First, the self-capacity of GSNO, **5e** and **5f** to modify the intracellular •NO pool was evaluated (Fig.4). GSNO aloneat a concentration starting from 50 µM was able to transfer •NO to HuAoSMCs by increasing nitrite ions and RSNOs amount (Fig. 4A) after one hour of incubation. Thus, for the following experiments, GSNO concentration will be fixed at 50 µM, as the first concentration inducing a significant modification of the intracellular •NO pool in HuAoSMCs. In the second part of this experiment, we investigated selenohydantoins **5e** (Fig. 4B) and **5f** (Fig. 4C) self-capacity to induce nitrite ions and RSNOs production in HuAoSMC. Neither **5e** nor **5f** were able to significantly modify the intracellular •NO pool in HuAoSMCs whatever their concentrations were

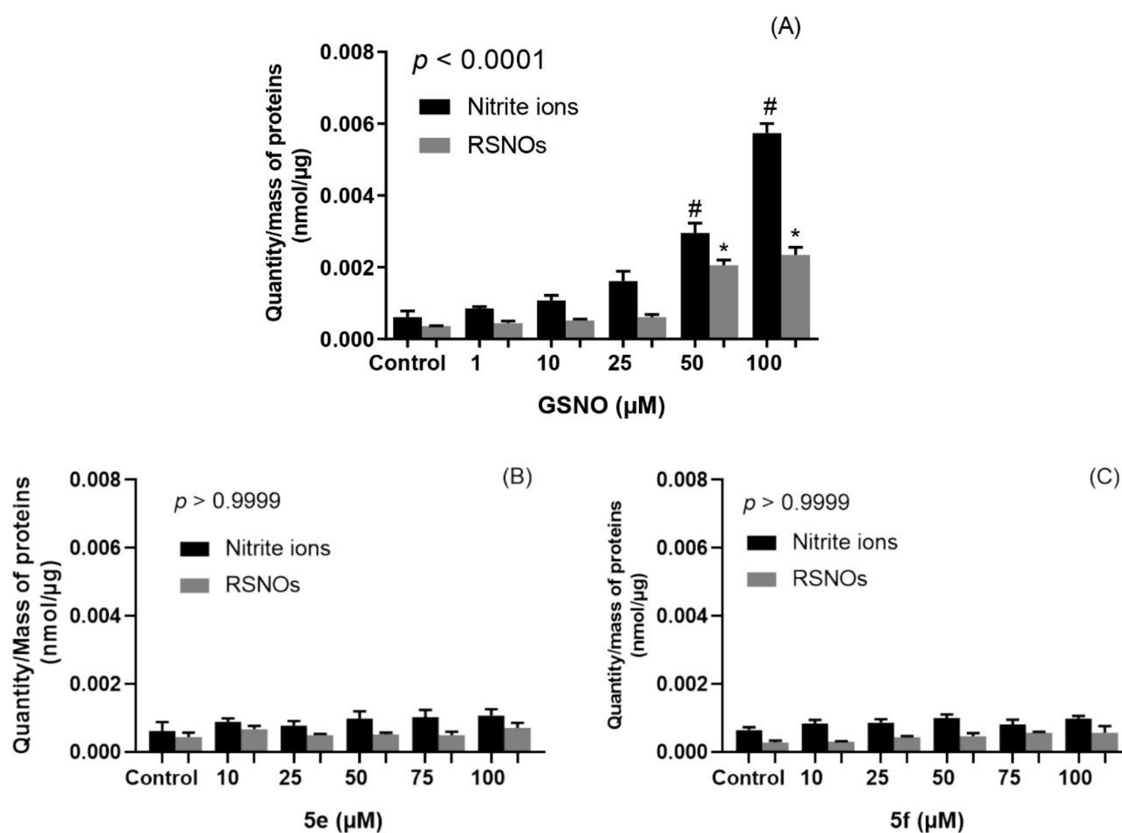


Fig. 4. Quantity of nitrite ions and RSNOs in HuAoSMCs, after one-hour incubation with GSNO (A), 5e (B) or 5f (C). Results are shown as means ± SEM of n=3 experiment done in triplicate, and compared with one-way ANOVA followed by a Bonferroni post-test. * *p* < 0.05 value *versus* Control

In the third part of this experiment, we investigated selenohydantoins derivatives (**5e** and **5f**) capacity to release, from GSNO, a bioavailable •NO (nitrite ions or RSNO concentrations) for HuAoSMCs. For that experiment, GSNO concentration was fixed at 50 µM and, **5e** and **5f** concentration was set at 2.5 µM, which is a catalytic concentration twenty times smaller than GSNO that correspond to the ratio studied in the experiment of *in tubo* redox catalytic release of •NONO (1 mM of GSNO and 50 µM of **5e** and **5f**). Moreover, in order to observe the downstream effects of a specific treatment, assess the temporal progression of events and identify specific time points when the effects are most pronounced, different sequences of treatments were studied such as co-incubation for one hour or sequential incubation (thirty minutes of GSNO followed by the addition of selenohydantoin for another thirty minutes or *vice versa*). In addition, we designed two GSNO controls such as thirty minutes or one-hour incubation of GSNO alone both suitable for each sequence of treatment (sequential **5e**/**5f**--GSNO or sequential GSNO--**5e**/**5f** and co-incubation, respectively). Interestingly, both sequential incubations and co-incubation of **5e** and GSNO increased intracellular nitrite ions when compared to GSNO thirty minutes incubation for **5e**--GSNO sequential incubation and compared to GSNO one-hour incubation for GSNO--**5e** sequential incubation as well as **5e** co-incubation with GSNO (Fig. 5A). This result suggests that **5e** potentiate and accelerate the release of •NO from GSNO in the form of nitrite ion. However, regarding intracellular RSNOs concentration only **5e** co-incubation with GSNO has a significant effect compared to the control and GSNO (one-hour incubation and thirty minutes incubation) (Fig. 5B). Here the kinetic of •NO release from GSNO does not seem to be the limiting factor for RSNOs formation. Whilst

5f either in co-incubation or sequential incubation with GSNO was able to increase the intracellular nitrite ions levels only when compared to GSNO thirty minutes (Fig. 5C) without succeeding in modifying intracellular RSNOs concentration (Fig. 5D). This result suggests that **5f** is less effective than **5e** in the catalytic release of a bioavailable •NO for smooth muscle cells such as seen in the *in tubo* experiment. Moreover, longer interaction time with GSNO might be required.

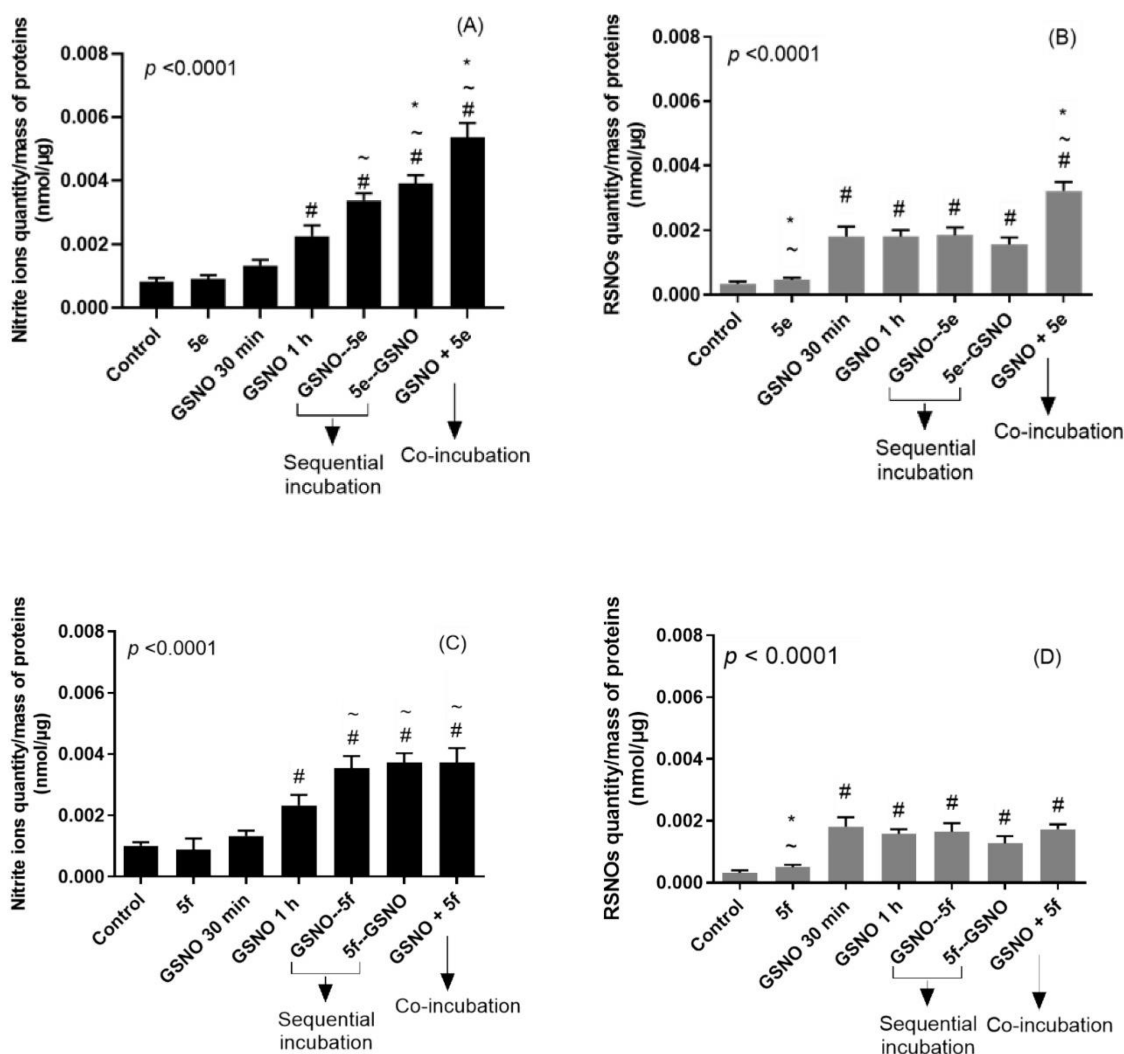


Fig. 5. Quantity of nitrite ions (A, C) and RSNOs (B, D) in HuAoSMCs, after one hour of total incubation with GSNO (50 µM) and/or 5e (2.5 µM) (A, B) and/or 5f (2.5 µM) (C, D). Results are shown as means ± SEM of n=4 experiments done in triplicate, and compared with one-way ANOVA (Bonferroni post-test). # *p* < 0.05 value *versus* Control, ~ *p versus* GSNO 30 min and * *p* 0.05 value *versus* GSNO 1 h.

3.2.2. Redox impact on intracellular reduced glutathione concentration

We have previously proven that GSH is an essential reducing agent in the GPx-like activity and the catalytic release of •NO by **5e** and **5f**. Therefore, **5e** and **5f** redox catalytic activity might affect the cell redox balance by consuming intracellular reduced GSH. In the counterpart, the presence of GSNO is a source of cysteine amino acids, which is the limited amino acid for GSH synthesis in cells. As a result, we explored if **5e** and **5f**, both individually and in combination with GSNO, might affect the intracellular concentration of GSH (Fig.6). **5e** and **5f** (Fig. 6A and, 6B respectively) did not affect intracellular GSH concentrations at those conditions. Whatever, the incubation conditions were, no variation in the intracellular concentration of reduced GSH was observed. This proved that in the presence of GSNO, the catalytic cycle engaged by **5e** and **5f** did not imbalanced GSH concentration also known as the cell redox buffer.

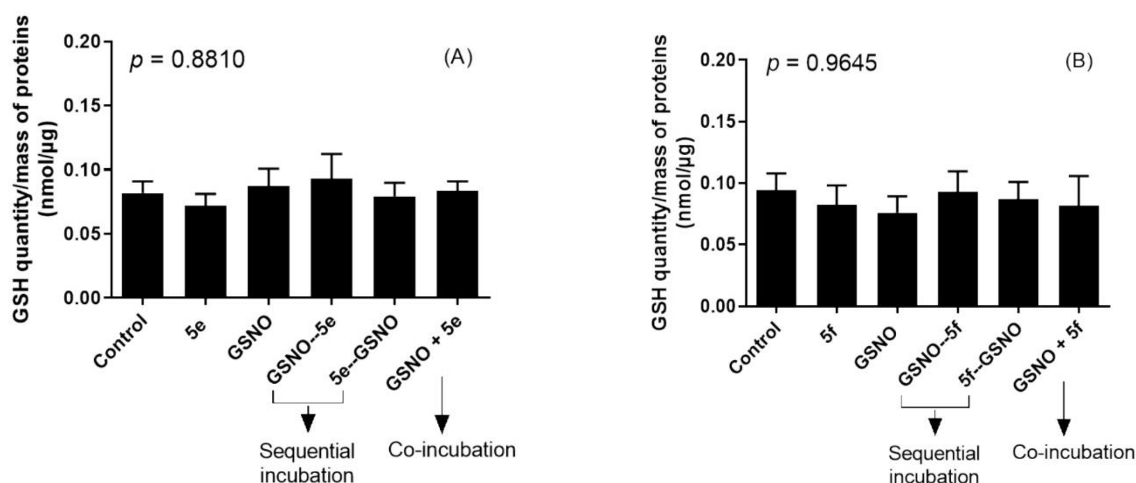
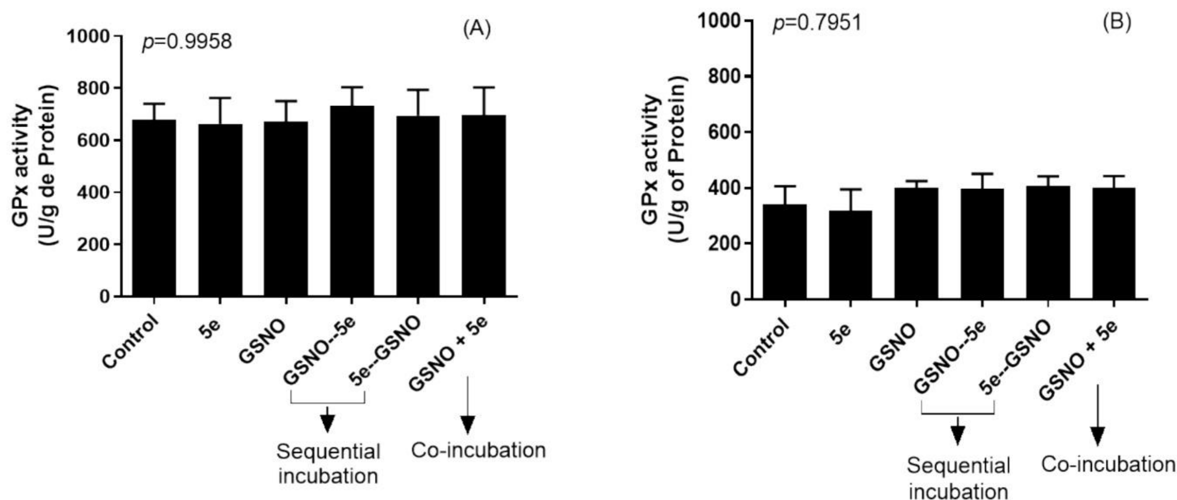


Fig. 6. Quantity of GSH in HuAoSMCs, after one hour of incubation with GSNO (50 µM) and/or 5e (2.5 µM) (A), and/or 5f (2.5 µM) (B). Results are shown as means ± SEM of n=4 experiments done in triplicate, and compared in one-way ANOVA.

3.2.3. Glutathione peroxidase activity *in vitro*

The ability of **5e** and **5f** to affect the cell GPx activity was evaluated using hydrogen peroxide (H_2O_2), an inorganic peroxide, and cumene hydroperoxide (CHP), an organic peroxide. In these set of *in vitro* experiments, the same incubation conditions as in other *in vitro* experimentations, respecting the same incubation time and concentrations were used. This investigation showed no interference or inhibitory effect of **5e** and **5f** on HuAoSMC GPx activity to reduce H_2O_2 (Fig. 7A and 7C) or CHP (Fig. 7B and 7D) for the studied conditions.



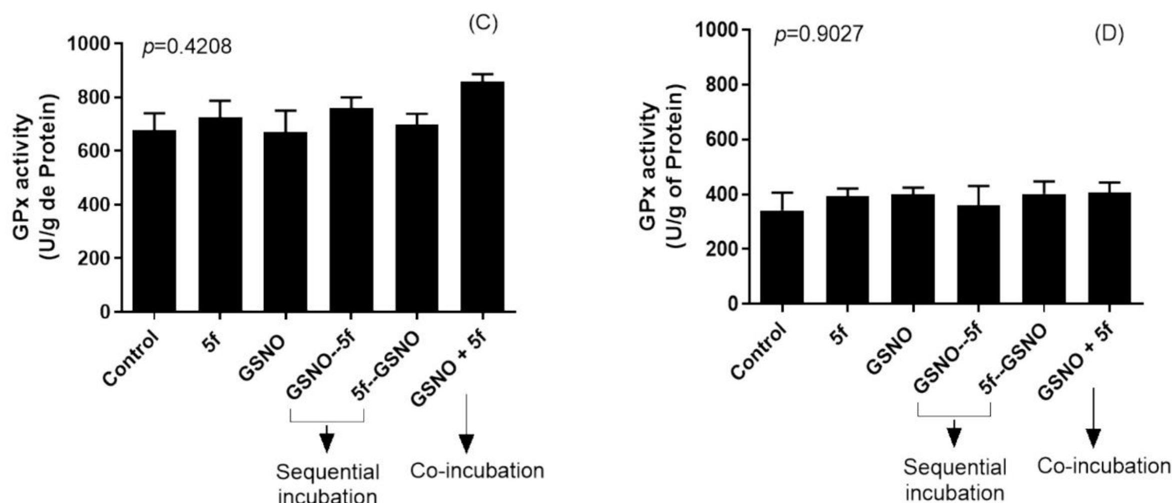


Fig. 7. Quantification of HuAoSMCs GPx activity after one hour of incubation with GSNO (50 µM) and/or 5e (2.5 µM) (A, B) and/or 5f (2.5 µM) (C, D) using hydrogen peroxide (A, C) and cumene hydroperoxide (B, D). Results are shown as means ± SEM of n=4 experiments done in triplicate, and compared with one-way ANOVA.

3.3. Vasorelaxation ex vivo

After demonstrating that **5e** and **5f** induce the release, from GSNO, of a bioavailable •NO for smooth muscle cells, we also wanted to know if they were able to catalyse the release of •NO from an intracellular pool of •NO and promote vasorelaxation. To that purpose, we used a previously designed model of •NO pool mobilization for aorta vasorelaxation [33]. However, we first checked if **5e** and **5f** alone exhibit vasorelaxant activity. Figure 7 shows that both selenohydantoins alone can induce a cumulative vasorelaxation, which increased with the concentrations tested, and the maximum of relaxation recorded on endothelium intact aortae at 10^{-5} M of **5e** or **5f** was 27 ± 4% (Fig. 8A) and 25 ± 4% (Fig. 8B), respectively.

However, those concentration response curves did not fit the Hill equation enabling to calculate any pharmacological parameter. Interestingly, the maximum of relaxation recorded on endothelium removed aortic rings at 10^{-5} M of **5e** was higher (31 ± 4%) (Fig. 8A) than for **5f** (23 ± 1%) (Fig. 8B)).

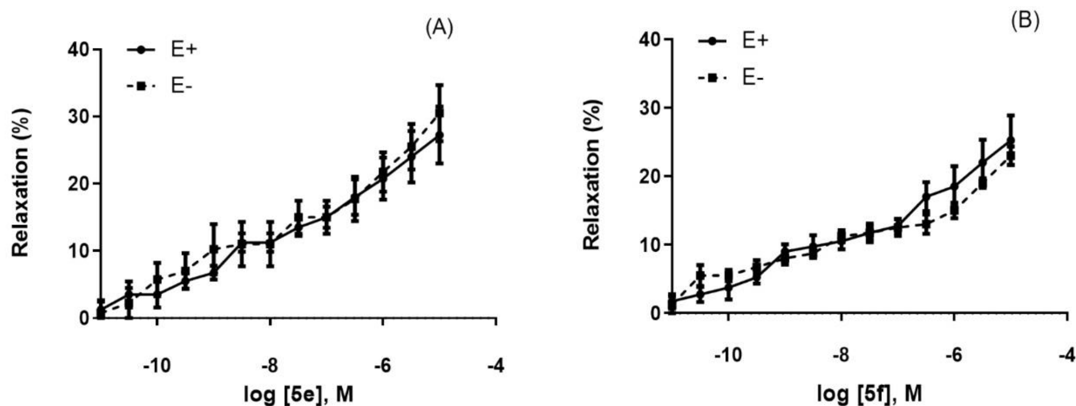


Fig. 8. Concentration-dependent response curves of 5e (A) and 5f (B) on endothelium-intact (E+) and endothelium-removed (E-) rat aortae precontracted with PHE 10^{-6} M. Results are presented as mean ± SEM of n =4 rings per group, from 4 different rats in each group.

The ability of **5e** and **5f** to mobilize a •NO stored, mainly composed of RSNOs, in aorta by pre-incubation with GSNO was then evaluated. To that purpose we used NAC as a positive control of •NO store mobilization. Indeed, according to previous studies, NAC alone did not show any vasorelaxant activities, but was able to mobilize the •NO store created by RSNO pre-incubation, resulting in vasodilation [33,34].

Further, we employed this protocol to compare if **5e** and **5f** were able to exhibit the same activity, bearing in mind that they had already shown vasorelaxation activity on their own. Figure 9 confirmed that 10^{-5}, 10^{-4} and 10^{-3} M of NAC were able to induce 22 ± 4%, 30 ± 4% and 18 ± 4% of vasorelaxation on endothelium intact aortic rings respectively, and 34 ± 7%, 36 ± 5% and 17 ± 2% of vasorelaxation on endothelium removed aortic rings respectively. Those results confirm that NAC is able to displace •NO from •NO stores formed after the preincubation with GSNO.

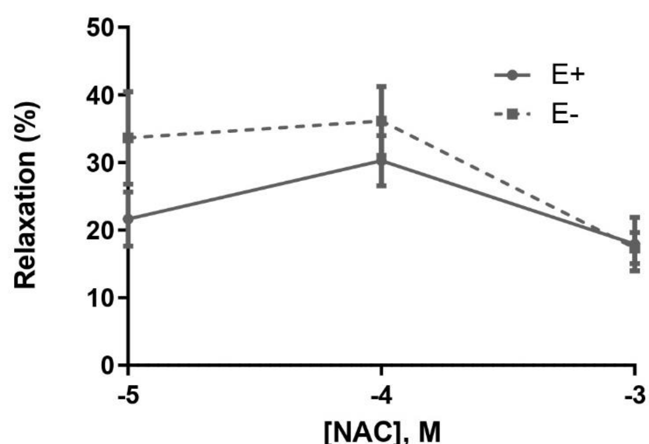
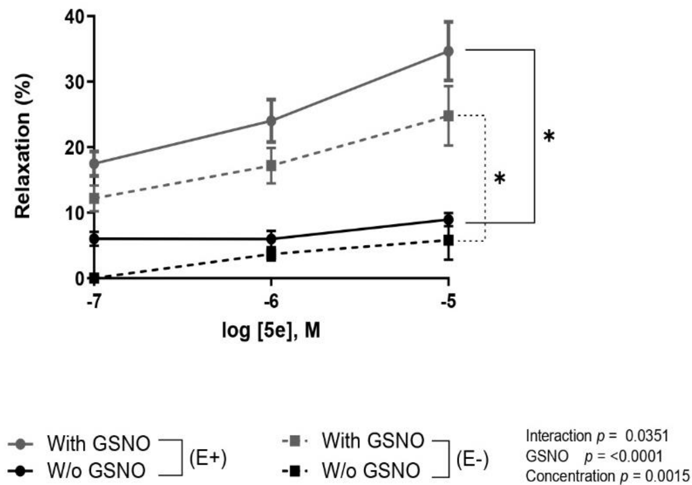


Fig. 9. •NO store mobilization by NAC for vasorelaxation of precontracted (10^{-6} M phenylephrine) endothelium-intact (E+) and endothelium-removed (E-) rat aortae after pre-incubation with 2 µM GSNO for thirty minutes and one-hour washout. Results are presented as mean ± SEM of n =7 rings per group, from 7 different rats in each group, and compared with Two-way ANOVA.

Using the same experimental conditions, 10^{-7}, 10^{-6} and 10^{-5} M of **5e** induced 17 ± 2%, 24 ± 3% and 35 ± 4% of vasodilation on endothelium intact aortic rings respectively, and 12 ± 2%, 17 ± 3% and 25 ± 5 % vasorelaxation on endothelium removed aortic rings respectively (Fig. 10A). These values of relaxation are much higher than 10^{-7}, 10^{-6} and 10^{-5} M of **5e** activity in the absence of •NO store (without GSNO, Fig. 10A) *i.e.* 6 ± 2%, 6 ± 2% and 9 ± 2% of vasorelaxation on endothelium intact aortic rings respectively, and 0,4 ± 1% and 6 ± 3% on

endothelium removed aortic rings, respectively. Whilst, **5f** at 10^{-7}, 10^{-6} and 10^{-5} M induced a 12 ± 1%, 12 ± 1% and 26 ± 4% vasorelaxation on endothelium intact aortic rings respectively, and 12 ± 2%, 18 ± 3% and 28 ± 4% vasorelaxation on endothelium removed and aortic rings respectively. These values of relaxation are higher than 10^{-7}, 10^{-6} and 10^{-5} M of **5f** activity in the absence of •NO store (without GSNO, Fig. 10B) *i.e.* 2 ± 1%, 4 ± 1%, and 4 ± 1% of vasorelaxation on endothelium intact aortic rings respectively, and 2 ± 1%, 4 ± 2% and 5 ± 1% vasorelaxation on endothelium removed and aortic rings, respectively. DMSO 0.02% alone (the solvent of **5e** and **5f**) did not induce any relaxant effect.

(A)



(B)

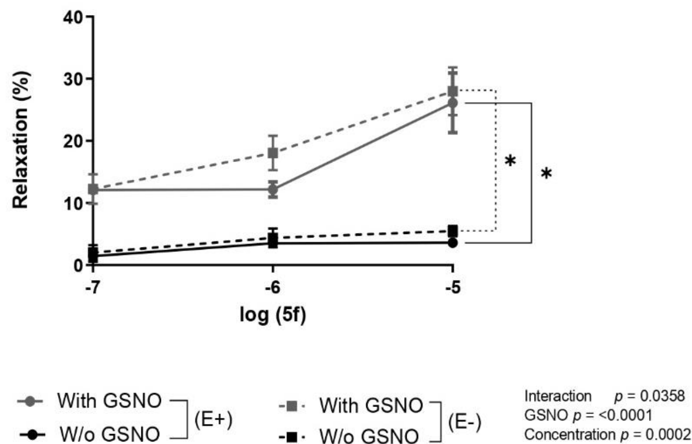


Fig. 10. •NO store mobilization by 5e (A) and 5f (B) for vasorelaxation of precontracted (10^{-6} M phenylephrine) endothelium-intact rat aortae (E+) and endothelium-removed (E-) rat aortae after preincubation or not with 2 µM GSNO for thirty minutes followed by one hour washing. Results are presented as mean ± SEM of n =3-9 rings per group, from 3 to 9 different rats in each group, and compared with Two-way ANOVA, (Bonferroni post-test). * *p* 0.05 value GSNO *versus* without GSNO.

Furthermore, Notably, **5e** and **5f** were more efficient than NAC. Their activities were observed at lower concentrations (10^{-7}, 10^{-6} and 10^{-5} M), in contrast to NAC which necessitated higher concentrations (10^{-5}, 10^{-4} and 10^{-3} M) for pronounced effects. This distinction is a crucial pharmacological aspect and is preferable for therapeutic considerations. In our experiment, **5e**, **5f** and NAC, met at a common concentration of 10^{-5} M, marking the highest for **5e** and **5f** and the lowest for NAC. Moreover, to gain better exploration of the differences between **5e**, **5f**, and NAC indirect vasorelaxant activities we compared them at their common concentration 10^{-5} M with or without GSNO either on endothelium intact and removed aortic rings (Fig. 11). We confirm here that preincubation with GSNO is mandatory to show **5e**, **5f** and NAC indirect vasorelaxant activity.

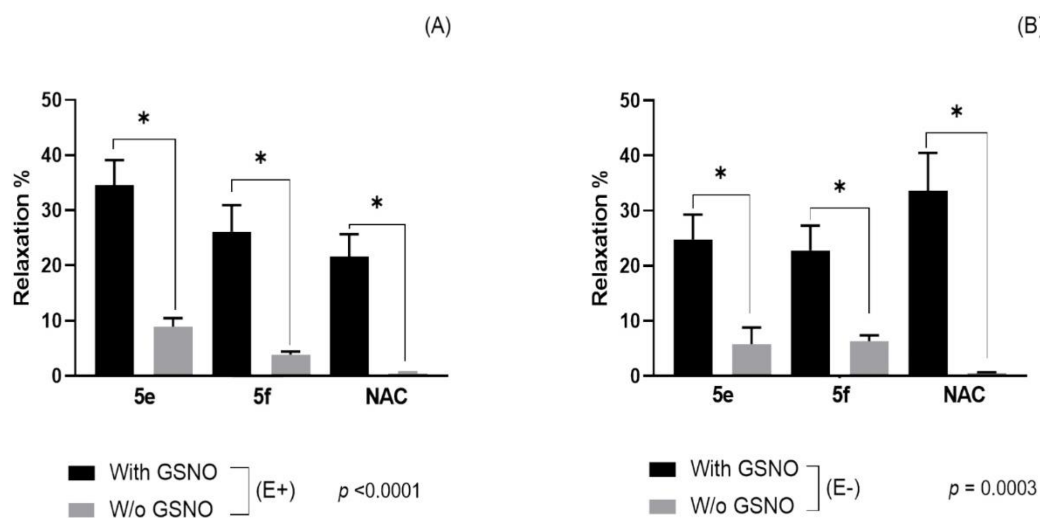


Fig. 11. •NO store mobilization by 5e, 5f and NAC (10^{-5} M) for vasorelaxation of precontracted (10^{-6} M phenylephrine) endothelium-intact rat aortae (E+) (A) and endothelium-removed (E-) (B) rat aortae after incubation or not with 2 µM GSNO for thirty minutes followed by one hour washing. Results are presented as mean ± SEM of n =3-9 rings per group, from 3 to 9 different rats in each group, and compared with one-way ANOVA, (Bonferroni post-test). * *p* 0.05 value GSNO *versus* without GSNO.

Interestingly, it was noticed that in aortic rings preincubated with GSNO for thirty minutes and washed, NAC induced a temporary vasorelaxation and allowed the vessel to re-contract again within fifteen minutes. And the activity was decreasing, which could be attributed to the consumption of •NO store. However, aortic rings treated with **5e** and **5f** had a long lasting vasorelaxation effect without any re-contraction for at least fifteen minutes and the activities were increasing simultaneously with the concentrations, which could be explained by the already proven activities of **5e** and **5f** (Fig. 12).

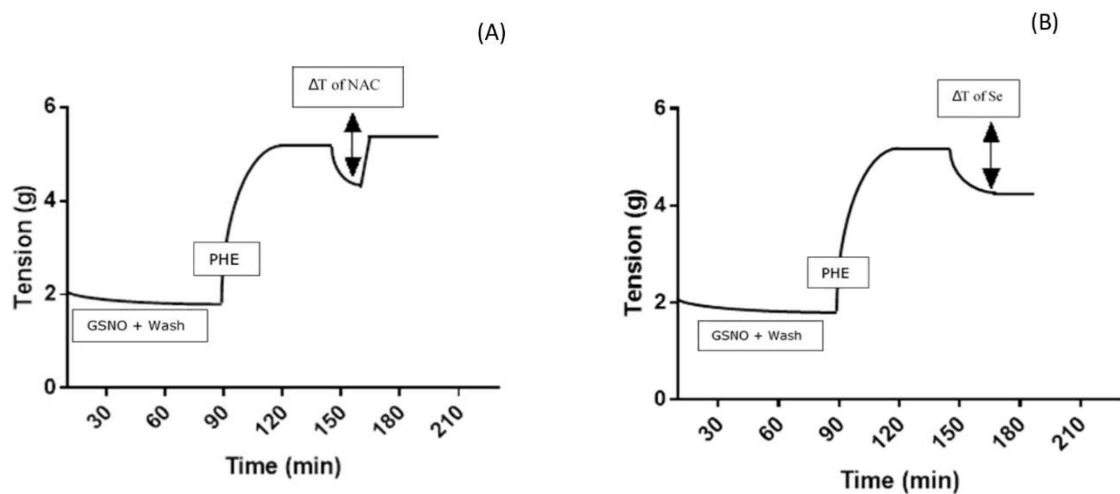


Fig. 12. Descriptive scheme of the impact, on aortic ring tension, NAC (A) or by 5e and 5f selenohydantoins (B), after pre-incubation with GSNO.

4. Discussion

This study aimed at exploring the catalytic activity and biological effects, targeting vascular homeostasis by modulating •NO bioavailability and vasorelaxation of newly synthesized selenohydantoins, inspired by selenoneine, a marine-originated molecule. In our prior published research, we highlighted the synthesis, antioxidant, and GPx-like activities of these compounds, as well as their cytocompatibility with HuAoSMCs. Notably, organoselenium molecules with GPx-like activity have been utilized previously for catalysing the release of •NO from RSNOs in the presence of a thiol, and enhancing protein *S*-nitrosation in endothelial cells [38].

We drew focus specifically on compounds **5e** and **5f**, distinguished by their significant GPx-like activity. T. This study investigated deeper into their ability to catalyse the release of •NO from GSNO in the presence of GSH as a reducing thiol. Indeed, GSNO is considered as the optimal choice among other RSNOs for our research, due to its ease of synthesis, biological abundance, compatibility with cellular uptake and metabolism and its prolonged stability which is required under physiological temperatures [58].

The *in tubo* catalytic experiments showed that **5e,** bearing a trifluoromethyl group (-CF_3), demonstrated the highest release activity of •NO from GSNO, in direct line with its highest GPx-like activity [54]. In the CF_3 group, the electron-withdrawing nature of fluorine atoms, directly attached to the carbon atom, induces an inductive effect that withdraws electron density from the ring, which plays an important role in enhancing these activities. In addition, the selenohydantoins were mainly active in the presence of GSH, assuredly, the presence of reducing agent such as GSH is essential to produce the active selenium species that are responsible for the decomposition of RSNOS, such as diselenide, selenosulfide, and selenol/selenolate (RSeH/RSe^-), and the possible SeNO intermediate [38,59]. This SeNO

intermediate could account for the observed increase in absorbance when the selenohydantoins interacted with GSNO and GSH *in tubo*. Moreover, recent studies have demonstrated the elementary-reaction processes involve an initial transnitrosation from RSNOs to selenols, resulting in the formation of *Se*-nitrososelenols (RSeNOs). These intermediates then rapidly form diselenide, releasing $^{\bullet}$NO in the process. Interestingly, it has been suggested that the formation of diselenide from RSeNOs to coincide with fast $^{\bullet}$NO generation. Unlike RSNOs, which do not readily undergo disulfide formation [60].

Furthermore, the use of organoselenium molecules in cardiovascular research is an active area of research, and have been used for the design of $^{\bullet}$NO-generating materials in particular when it can mimic the catalytic activity of GPx activity, which is able to decompose RSNOs, to achieve long-term, stable, $^{\bullet}$NO release. Therefore, promoting re-endothelialisation, controlling thrombosis, and reducing intimal hyperplasia *in vivo* [61,62]. Thus, building on the concurrent research and based on our findings, **5e** and **5f** were further evaluated for their effect on intracellular $^{\bullet}$NO and *S*-nitrosation, which are assessed indirectly by quantifying intracellular nitrite ions and RSNO levels. Nitrite ions are stable metabolite of $^{\bullet}$NO, it serves as a reliable indicator of $^{\bullet}$NO production and bioavailability. It can be quantified using various assays, and provides insights into the overall production and release of $^{\bullet}$NO within a biological system, including cells or tissues. While measurement of RSNOs indicates *S*-nitrosation reactions, providing information about the processes of protein *S*-nitrosation within the cellular environment [63–69].

HuAoSMCs were selected as an *in vitro* relevant model, the choice of this cell type is interesting given its role in vascular research and its tolerance towards the selenohydantoins synthesized [54,70–72]. Research has already shown the ability of GSNO to increase the levels of intracellular RSNOs and nitrite ions, in addition to the ability of GSNO to protect reduced protein thiols from oxidative stress, making them again available to react with $^{\bullet}$NO. Indeed, an RSNOs

increase is observed under oxidative conditions [57,73,74]. Protein *S*-nitrosation directly modulates protein function, and is also a form of '•NO store' from which biologically active •NO can be released as and where needed. Therefore, investigating •NO stores formation and bioavailability could be valuable for evaluating the therapeutic efficiency of •NO donors [75–77]. On one hand, **5e** co-incubated with GSNO for one hour or in a sequential pattern (**5e** incubated for thirty minutes then followed by GSNO for another thirty minutes) exhibited a significant increase in intracellular nitrite ions compared to GSNO one-hour incubation, on the other hand, all conditions of co-incubation and sequential incubations significantly increased nitrite ions when compared to GSNO thirty-minutes incubation, suggesting the benefit of the longer incubation with **5e**. H. However, only the co-incubation of **5e** and GSNO for one hour was able to increase the intracellular RSNOs compared to GSNO alone incubated for one hour, suggesting the capability of **5e** to influence •NO levels in particular and *S*-nitrosation when coupled with GSNO. This finding also agrees with the notion that **5e** exhibited superior activity in releasing •NO from GSNO *in tubo* and ability of organoselenium molecules to enhance the bioavailability of •NO and protein *S*-nitrosation [37,38,62,78–80]. The other experiment with **5f** showed an increase of nitrite ions in the co-incubation and sequential incubations only when compared to GSNO incubated for thirty minutes, did not exhibit any increase of RSNOs intracellular concentrations compared to GSNO (thirty minutes and one hour) alone suggesting the need for a longer interaction time between GSNO and **5f** to induce significant activity and also confirms that there is a synergic interaction between **5e** and GSNO to induce the catalytic release of •NO.

Furthermore, it is known that GSH is an important regulator of the cell redox state, and as already seen, it plays a pivotal role in the catalytic cycle of GPx activity and •NO release from GSNO [81,82]. In a previous study, it was demonstrated that GSNO at 50 μM did not affect intracellular GSH levels under normal conditions [57]. The protection provided by GSNO to

intracellular protein thiol (-SH) groups happens *via* protein *S*-nitrosation, and can also contribute to the maintenance of intracellular redox balance and offer protection against oxidative damage mediated by ROS [83]. Furthermore, this was also seen in our experiment, where no significant changes observed in intracellular GSH levels, which remained stable for the different studied incubation conditions. That in essence is not surprising since the normal GSH intracellular levels are relatively high and it may have not been affected by our designed study, in addition, the antioxidant activity of the studied molecules could also contribute in controlling oxidative stress and cell redox homeostasis, therefore protecting the GSH pool [84]. Therefore, selenohydantoin derivatives have the ability to release a bioavailable •NO for smooth muscle cells without affecting their intracellular redox balance regarding intracellular GSH concentration.

Moreover, •NO and its donors were believed to have an inhibitory effect on GPx activity, because •NO could modify the activities of several enzymes, where the SeNO modification of selenoproteins may lead to their degradation through oxidative deselenation, therefore, it was of our interest to determine if the conditions studied in our *in vitro* experiments could affect the GPx activity [85–88]. Our investigation demonstrated that GSNO at 50 μM have no inhibitory activity for intracellular GPx like activity, and neither any of the incubation conditions altered the levels of GPx activity in HuAoSMCs. Additionally, **5e** and **5f** did not exhibit an added GPx activity to reduce H_2O_2 or CHP *in vitro*. In fact, GPx mimics, such as ebselen, have shown low activity *in vitro* due to the lack of a GSH binding site, indeed, chemical modifications could improve the GSH binding ability [89–92]. This may also be attributed to the complex nature of cellular redox homeostasis and the specific conditions of incubation time and concentrations employed in our experiments. Further investigations into the underlying molecular mechanisms are needed to elucidate the observed outcomes. Moreover, organoselenium molecules such as selenohydantoins have been previously proved to have cardioprotective effects, and have great

potentials in treating cardiovascular illnesses [54,93]. Indeed, the *ex vivo* experimentation revealed the ability of **5e** and **5f** to induce a cumulative vasorelaxation in endothelium intact and endothelium removed isolated rat aortic rings. The observed activities may be attributed to the ability of **5e** and **5f** to release •NO catalytically from RSNOs and to their antioxidant properties. Indeed, organoselenium molecules are found to increase the expression of important antioxidant enzymes, which can help to reduce oxidative stress and to improve vascular function *via* •NO release pathways and endothelial function improvement [75,94–97]. Moreover, these complex and indirect pathways could explain the non-Hill response, in which indicates a lack of direct cooperativity in the binding of the molecule of interest to a receptor or to an enzyme. For instance, the activation of the sGC/cGMP pathway, could be achieved particularly through the release of a physiological •NO pool that activates the •NO-sGC pathway. Interestingly, **5e** and **5f** also exhibited activities on endothelium removed aortic rings which also suggests their ability to compensate at some endothelial functions. This notion corresponds with a previous study that suggested that selenium can restore a normal metabolic activity and improves vascular responses and endothelial dysfunction in diabetic rats by regulating antioxidant enzyme and •NO release [98]

Previous studies have demonstrated that RSNOs can establish a •NO reservoir available for vasorelaxation [33,76,99]. Notably, GSNO is recognized as a key player in the cardiovascular system. It not only induces protein modifications, particularly through *S*-nitrosation, but also acts as the primary biological •NO store and transporter, contributing to •NO vasodilatory activities [58,99]. The presence of •NO vascular stores has been associated to the vasorelaxant effects for molecules like NAC, where •NO from intracellular RSNO can then be released, by NAC allowing sGC activation for vasorelaxation [33]. Notably, NAC did not exhibit vasorelaxant activities without prior incubation with RSNOs [33,99]. Consequently, we investigated the interaction of **5e** and **5f**with •NO vascular stores and their ability to displace •NO and allow its

release from *S*-nitrosated peptides or proteins to induce vasodilation. GSNO was employed to create these •NO stores in endothelium intact and endothelium removed isolated rat aortic rings, which were later subjected to **5e** or **5f**. Interestingly, this phenomenon led to an enhanced vasorelaxant activity compared to rings not preincubated with GSNO in both endothelium intact and endothelium removed aortic rings. Indeed, this implies that **5e** and **5f** interacted with •NO stored in the vessel, and displaced it through their catalytic activities for further vasorelaxant activities (Fig. 13). Furthermore, we may suggest that these molecules through the created •NO store in smooth muscle cells might also restore the impaired role of •NO production and endothelial function via their antioxidant •NO releasing activities [19,23,24,37,62,75,76,98–106]

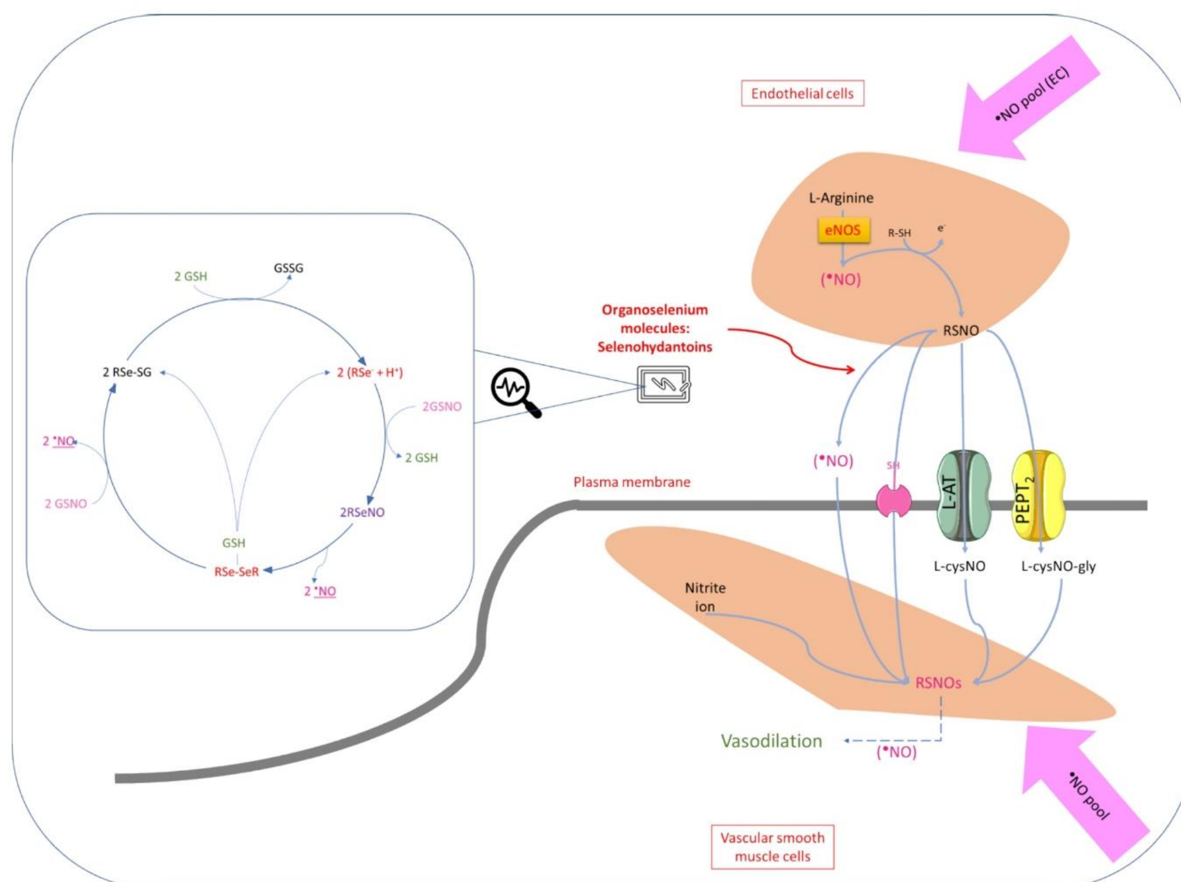


Fig. 13. Storage and transport of nitric oxide (•NO) as *S*-nitrosothiols (RSNOs) and the effects of selenocompounds on •NO release from RSNOs, and a proposed mechanism of

new selenohydantoins (*e.g.* 5e and 5f) to release •NO *via* their catalytic activities. The catalytic cycle shows •NO release from *S*-nitrosoglutathione (GSNO) by different forms of organoselenium molecules (RSe), in the presence of glutathione (GSH).

***Se*-nitrososelenol (RSeNOs), Reduced glutathione oxidized glutathione (GSSG), Selenone (RSe), Selenolate (RSe^-) and Selenenyl sulfide intermediate (RSe-SG). *S*-nitrosocysteine (L-cysNO), L-cysNO-glycine (L-cysNO-gly)L-type amino acid transporter (L-AT) or dipeptide transporters (PEPT2). Extracellular •NO is transported to the cytoplasm *via* membrane thiols (transnitrosation).**

5. Conclusions

This study shed a light on the multifaceted biological activities of selenohydantoins, showcasing their catalytic activity in releasing •NO *in tubo, in vitro* and *ex vivo.* Indeed, **5e** and **5f** exhibited interesting activities in releasing •NO from GSNO *in tubo*, these activities were employed to enhance intracellular •NO levels and *S*-nitrosation, which was manifested in increasing nitrite ions and RSNOs by **5e** and GSNO co-incubation in particular. Moreover, **5e** and **5f** as well as GSNO exhibited their activities without disturbing the cellular redox balance and GSH intracellular levels. In addition, neither GSNO nor selenohydantoins inhibit cell GPx activity. Moreover, **5e** and **5f** showed an intriguing ability to induce vasorelaxation *via* indirect pathways that involves antioxidant and •NO releasing activities, which confirms the findings observed *in tubo* and *in vitro*. The responses observed emphasize the complexity of redox processes, and the multi-layered warranting future investigations into the precise mechanisms governing these interactions. Therefore, those two selenohydantoins can be used to increase •NO release either in the blood stream and from the vascular wall to promote vasorelaxation. The potential implications of these findings in the context of cardiovascular health make these selenohydantoins in particular **5e** a promising candidate for further exploration and development.

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3.2 *Conclusions*

The diverse biological activities of selenohydantoinshave been previously evaluated, however, in this study we have showcased for the first time the activities of new selenohydantoins by demonstrating their catalytic activity in releasing •NO in different experimental settings, including *in tubo*, in vitro *and ex vivo*. In particular, molecules **5e** and **5f** showed interesting activities in releasing •NO from *GSNO in tubo*. These activities were used to enhance intracellular •NO levels and *S*-nitrosation, as evidenced by the observed increase in nitrite ions and RSNOs upon co-incubation with 5e and GSNO. Furthermore, both **5e** and **5f**, together with GSNO, exerted their activities without disturbing the cellular redox balance or intracellular GSH levels. Notably, neither GSNO nor selenohydantoins inhibited cellular GPx activity.

Furthermore, molecules **5e** and **5f** showed an intriguing ability to induce vasorelaxation *via* indirect pathways involving antioxidant and •NO-releasing activities, corroborating the findings observed *in tubo* and *in vitro* experiments. These responses highlight the complexity of redox processes and warrant further investigation into the precise mechanisms governing these interactions.

Therefore, these two selenohydantoins hold promise for increasing •NO release both in the bloodstream and from the vascular wall to promote vasorelaxation. The potential implications of these findings, particularly for molecule **5e**, make it a promising candidate for further investigation and development, in the context of CVDs associated with oxidative stress and endothelium dysfunvtion.

4 General discussion

Cardiovascular diseases (CVDs) remain a serious challenge to global health, demanding a continual search for innovative therapeutic strategies to address their complex etiology [1,2]. On one side, oxidative stress, an imbalance between ROS production and the antioxidant defence mechanisms such as GPx, plays a crucial role in CVDs initiation and progression with the decrease in •NO bioavailability and endothelium functions impairment. On the other side, antioxidants are essential in neutralizing ROS and maintaining redox balance. Furthermore, organoselenium molecules are an important example of antioxidants, this role could be attributed to their redox-modulating potentials and GPx-like activity. The potential therapeutic applications of organoselenium molecules include disease prevention, especially in conditions associated with oxidative stress, and their anti-inflammatory effects further enhance their significance. This study shed a light on the link between the antioxidant properties of organoselenium molecules and their •NO release activity from •NO storage forms. Our investigations focused on the potential of recently synthesized selenohydantoins inspired by selenoneine [3–5]. Selenoneine, first isolated from tuna fish, is a unique derivative of seleno-histidine with a selenium atom on the imidazole ring. This natural organoselenium molecule exhibits significant antioxidant activity, suggesting a broad spectrum of therapeutic applications. However, due to its hydrophilic nature, isolation from biological samples could be challenging, in addition, its multistep chemical synthesis is cumbersome, resulting in very low yields. Recent efforts have focused on synthesizing organoselenium molecules with antioxidant and therefore redox modulation capabilities. selenohydantoin derivatives are noteworthy examples, sharing a fundamental seleno-imidazole ring structure with selenoneine. Thus, our study aimed to bridge the gap between selenoneine properties and more accessible selenohydantoin derivatives. Notably, their relatively straightforward synthesis with good yields make them prominent target molecules, with a focus on their antioxidant and GPx-like activities to counteract oxidative stress. Furthermore, selenohydantoin activities aimed to increase •NO bioavailability and promote its release from RSNOs, as well as improving endothelial function and overall cardiac health [1,2,6–13]

The newly synthesized selenohydantoin derivatives, featuring a selenium atom on the imidazole ring, demonstrated considerably better solubility than ebselen, and less water solubility than selenoneine, which is important for handling and bioavailability. Moreover, they revealed a remarkable spectrum of reducing and radical scavenging activities. Notably, their efficacy exceeded those of reference antioxidant molecules such as glutathione (GSH), and ebselen, the flagship reference GPx mimics [3,14–18]. Especially, **5e** with a trifluoromethyl (-CF_3) substitution exhibited comparable GPx-like activity to

ebselen in reducing hydrogen peroxide (H_2O_2), and superior GPx-like activity than selenocystamine, which is known for its potent antioxidant properties [3,19].

Moreover, the link between GPx activity and •NO release has been a subject of scientific investigation. For instance, a deficiency in GPx3 has been associated with a decrease of bioavailable •NO, suggesting a potential link between GPx3 activity and •NO bioavailability [20]. Similarly, another study discussed the release of •NO from RSNOs mediated by a GPx-mimic, indicating a connection between GPx-like activity and the release of •NO from RSNOs [21,22]. Numerous studies in the literature have already shown the ability of different organoselenium molecules to release •NO from RSNOs [23–27]. Therefore, as the decrease of •NO bioavailability if often corelated with CVD development, we investigated selenohydantoin •NO release activity from RSNOs notably from GSNO in the presence of GSH as a reducing agent to attest the implication of a redox cycle mainly linked to their GPx-like activity. Among newly synthesized selenohydantoins, both **5e** and **5f** exhibited •NO release activity, with **5e** activity even higher than selenocystamine, a reference organoselenium molecule that has been already employed in •NO release from GSNO [3,23–27,30–32]. In fact, the strategic introduction of a (-CF_3) substitution in **5e**, despite its non-physiological nature, significantly ameliorated **5e** activities (either GPx-like and •NO release). CF_3 is known for its electron-withdrawing effect powered by fluorine atoms directly attached to the carbon atom inducing an inductive effect that withdraws electron density from the ring [3,33,34]. These differences originate from long-range inductive effects to the selenium, a phenomenon that could stabilize the selenosulfide intermediate during the catalytic cycle [35]. Moreover, a recent study has provided the proof of the formation of SeNO intermediate species in the reaction mechanisms of seleno-catalysed •NO generation from RSNOs and the rapid simultaneous formation of a diselenide afterwards, in the presence of a reducing thiol (GSH) [41]. Moreover, our theoretical calculations also showed a distribution of selenone/selenolate forms in the presence of basic species, which results in the formation of selenolate (RSe^-) since hydantoins have a highly acidic proton predominating over the selenol form at physiological pH values [3,36–38]. Moreover, selenolate is highly reactive, and direct antioxidant actions of seleno-molecules arise from the nucleophilic properties of selenolate [3,5,23,39,40]. However, selenohydantoins exhibited less activity with NACNO and SNAP than with GSNO. This suggests that GSH coupling with its own nitrosated form, GSNO, could be more straightforward than the coupling with NACNO or SNAP, additionally, the latter molecules could be potentially exerting an inhibitory effect on molecules with GPx-like and catalytic activities [23,26,30–32]. Moreover, the literature previously investigated the interaction between SNAP and GPx. Various studies have demonstrated the inhibitory effect of SNAP on GPx, likely attributable to the modification and oxidation of crucial residues such as cysteine (Cys^{74} or ^{91}) and selenocysteine ($Secys^{45}$). The selenol groups within the active sites of the (Secys) residue are particularly implicated in this process,

ultimately leading to enzyme deactivation through the formation of a selenenyl sulfide bridge. This phenomenon may explain the observed lack of activity with SNAP in our *in tubo* experiment[41–44].

The use of organoselenium molecules in cardiovascular research is an active area of research, they have been already used to generate •NO in particular when it can mimic the catalytic activity of GPx, which is able to decompose RSNOs, to achieve long-term and stable •NO release [28,29]. The heightened potential of selenohydantoins coupled with their cytocompatibility with HuAoSMC, made them promising candidates for further exploration as antioxidant and central player in the catalysis of •NO pool formation and release. Notably, molecules **5e** and **5f** emerged as frontrunners and were chosen for further investigations in their potential to help in •NO pool formation *in vitro* and •NO pool release *ex vivo*. Both candidates exhibited capacity to release, from GSNO, a bioavailable •NO for HuAoSMC materialized by an increase in intracellular nitrite ion concentration, the stable form of •NO in aqueous medium. However, only **5e** was able to increase the intracellular RSNO concentration suggesting that there is a synergic interaction between **5e** and GSNO to induce *S*-nitrosation and the catalytic release of •NO. This finding also correlates the superior activity of **5e** in releasing •NO from GSNO *in tubo* [25,30–32,45,46]. The increase of the intracellular •NO pool suggest an increase in bioavailable •NO, and its signalization through protein *S*-nitrosation, crucial aspects in regulating vascular tone and function [7,45,47–54]. As already discussed, GSH plays a pivotal role in GPx activity and •NO release from GSNO in the catalytic cycle. Our experiments, revealed stable intracellular GSH levels across various incubation conditions *in vitro*, and consistent with the intracellular high GSH levels. The antioxidant activity of **5e** and **5f** further aids in controlling oxidative stress, maintaining cell redox homeostasis, and safeguarding the GSH pool, alongside the ability to release bioavailable •NO for smooth muscle cells without disturbing intracellular redox balance.

Moreover, the potential inhibitory effect of •NO and its donors on GPx activity was already discussed, where SNAP was shown to induce the inactivation of GPx, probably through SeNO modification and oxidation of the Sec residue and the formation of a selenenyl sulfide bridge, this inhibitory effect of SNAP on GPx was reversed by dithiothreitol (DTT), suggesting that the SeNO modification of selenoenzymes can be easily reversed by reducing thiols [41–43,55–58].

Our investigation revealed that GSNO did not inhibit neither HuAoSMC GPx activity nor **5e** and **5f** GPx-like activity that was manifested in their catalytic activity in increasing the intracellular •NO pool. Nevertheless, the conditions utilized to modulate •NO levels and *S*-nitrosation did not disrupt intracellular GSH homeostasis or GPx activity, which unveils a nuanced mechanism. This suggests a potential dual role in maintaining cellular redox balance while concurrently enhancing •NO bioavailability, which could target redox signalling pathways [59,60].

Finally, to achieve our goal of utilizing selenohydantoins activities to improve •NO bioavailability and overall vascular health[48,61–67], we investigated their ability to catalyse •NO release from a •NO pool stored in an aortic wall *ex vivo*. Therefore, we first demonstrated that **5e** and **5f** have an intrinsic vasorelaxant capacity of around 30% both on endothelium intact or removed aortic rings precontracted. This suggest that **5e** and **5f** vasorelaxant activity is an endothelium-independent phenomenon, which is not mediated by the stimulation of a receptor or an enzyme as concentration response curves did not fit the Hill equation [18,26,66,68–71]. Additionally, considering the insights gained regarding the impact on •NO levels *in tubo* and *in vitro*, further experiments were necessary to evaluate whether these activities are achievable *ex vivo* particularly with a focus on **5e** and **5f**. [23,25–27,30]. Thus, the aortic rings were either pre-incubated with GSNO to form the •NO store, or left untreated as negative controls [53,63,66,72]. NAC was employed as a positive control, since in prior studies it exhibited vasorelaxant activities only *via* mobilizing •NO from its stores, then all aortic rings were contracted with PHE [53,66,73–75]. Interestingly, the aortic rings pre-incubated with GSNO showed more vasorelaxation that the ones without an induced •NO store. Which suggests that **5e** and **5f** were able to release (denitrosate) •NO from nitrosated molecules /•NO store formed by preincubation with GSNO, for both endothelium intact and removed aortic rings [53,63,66,72]. The observed effects, suggestive of a dual mechanism involving antioxidant capabilities and •NO release, underscore their potential impact on vascular function and endothelial dysfunction [11,61,69,74,76–78]. The ability of these molecules to modulate •NO from vascular stores, particularly in preincubated aortic rings, adds a layer of complexity, indicating their potential as direct vasodilators by mobilizing •NO and redistributing it where needed.

After all, the newly synthesized **5e** and **5f** selenohydantoins with their antioxidant and •NO release activity both linked with their GPx-like catalytic activity open a great avenue as potential candidates for CVD treatment.

5 Conclusions and Perspectives

This study provided a new series of promising selenohydantoins, a class of organoselenium molecules inspired by the elusive selenoneine, with enhanced solubility, stability and multifaceted impacts on oxidative stress, •NO bioavailability, and vascular function. **5e** in particular, emerged as promising candidate for therapeutic interventions in cardiovascular health and redox modulation.

While our study presents promising findings, the complexity behind it could be limiting. One aspect deserving attention is the examination of the long-term stability of the newly synthesized selenohydantoins. This should extend to diverse laboratory settings and experimental conditions to ensure the robustness and reproducibility of our results over time and under varied circumstances.

Likewise, it is crucial to understand the intricate nature of cell redox homeostasis, and the huge impact of organoselenium molecules on it. Research on selenium in redox biology has a well-established foundation, especially in the context of selenoproteins. Nonetheless, the potential for selenium's redox activity and its susceptibility to oxidation require a careful examination of its effects on cell redox status. Additionally, its interactions with sulfur-containing molecules, including GSH and RSNOs, call for careful evaluation. These considerations are essential for advancing our understanding of selenium's role in redox biology and its potential implications for health.

While *in tubo* experiments undoubtedly establish an important research foundation, the nature of *in vitro* or *ex vivo* experiments holds additional complexity due to the presence of different cell components and mechanisms. Therefore, significantly impact the outcomes. Moreover, it is essential to recognize that the lack of specific binding sites *in vitro* or *ex vivo* can lead to an underestimation of the true physiological impact of these selenohydantoins, and diminish observed activities, as seen with the GPx-activity (*in tubo versus in vitro)*.

Furthermore, monitoring the different intermediates resulting from selenium interactions is crucial. This includes tracking various forms of selenium (*i.e.* selenolate, selenol, selenone, diselenide and selenenic acid), observing selenosulfide intermediates, and identifying potential (SeNO) species that could be also contributing to the catalytic release of •NO. As we already discussed in a previous review , numerous spectroscopic methods, such as UV-Vis and Nuclear Magnetic Resonance (NMR) spectroscopy, mass spectrometry, and high-performance liquid chromatography (HPLC) are indeed valuable for detecting different selenium species [79].

Moreover, the specific experimental conditions employed, including factors like incubation time and concentrations, warrant careful consideration. Such as seen *in vitro*, where longer incubation periods of **5e** and **5f** could provide additional values, where it was observed that a longer interaction between

5e and GSNO significantly modify the intracellular •NO pool in HuAoSMCs. In addition, investigating different concentrations of the selenohydantoins could also provide an in-depth understanding to their concentration/activity relationship. Further exploration into the interaction with different thiols is also needed to evaluate the ability of these selenohydantoins to release •NO from different RSNOs. Moreover, the observed complex activities *ex vivo* could explain the non-Hill response, in which indicates a lack of direct cooperativity in the binding of the molecule of interest to a receptor or to an enzyme. Therefore, to explore the involvement of the sGC/cGMP pathway, it is recommended to employ a specific sGC inhibitor, such as H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), known for its potent and selective inhibition of •NO-stimulated GC activity. Concurrently, investigating the modulation of different enzymes, including nicotinamide adenine dinucleotide phosphate (NADPH) oxidases and endothelial nitric oxide synthase, is essential for evaluating the expression of vasorelaxation activity and the antioxidative potential. Notably, the observed activities of **5e** and **5f** on endothelium-removed aortic rings suggest the involvement of both endothelium-dependent and independent pathways, this is commonly achieved by employing endothelium-independent agents as positive controls (*e.g.* sodium nitroprusside and nitroglycerin) that act directly on smooth muscle cells and cause vasorelaxation.

Furthermore, *in vivo* Investigations could validate the findings. Indeed, studying the molecules' effects in living organisms will provide a more realistic representation of their potential therapeutic impact and help close the gap between laboratory research and clinical applications. Investigating the long-term effects is essential to ensure their potential as therapeutic agents, for instance, toxicity studies to evaluate the adverse effects of the synthesized selenohydantoins in animal models, in order to assess both acute and chronic toxicity, such as mortality, and any signs of organ damage. In the same manner, studies to examine whether prolonged exposure to the organoselenium molecules may lead to the development of tumours or carcinogenic effects is crucial. Moreover, it is equally important to evaluate their metabolic stability (*i.e.* absorption, distribution, metabolism, excretion, and interaction with other medications).

Finally, organoselenium molecules have been already used for the design of •NO-generating materials in particular the GPx mimics, which are able to decompose RSNOs, thus, achieve long-term, stable, •NO pool. For instance, developing materials for sustained •NO release involves encapsulating or immobilizing organoselenium molecules in a suitable carrier, such as a biocompatible polymer or other matrices. Techniques such as microencapsulation, embedding in hydrogels, or nanoparticle formulation may be explored. Indeed, nanotechnology can be utilized to develop nano-based formulations for the delivery of organoselenium molecules (*i.e.* selenohydantoins), potentially enabling their use in sustained •NO release methods. These formulations can enhance the stability,

controlled release kinetics, and biocompatibility of the studied molecules, thereby facilitating their application in cardiovascular research. Nano-selenium and its nanomedicine applications highlight the potential for innovative administration methods and improved bioavailability and safety.

Zusammenfassung und Perspektiven

Diese Studie führte zu einer neuen Reihe vielversprechender Selenohydantoine, einer Klasse von Organoselenverbindungen, die vom schwer fassbaren Selenonein inspiriert sind und eine verbesserte Löslichkeit, Stabilität und vielfältige Wirkungen auf oxidativen Stress, die •NO -Bioverfügbarkeit und die Gefäßfunktion aufweisen. Insbesondere **5e** hat sich als vielversprechender Kandidat für therapeutische Interventionen im Bereich der kardiovaskulären Gesundheit und der Redoxmodulation erwiesen.

Obwohl unsere Studie vielversprechende Ergebnisse liefert, könnte die Komplexität, die sich dahinter verbirgt, einschränkend wirken. Ein Aspekt, der Aufmerksamkeit verdient, ist die Untersuchung der Langzeitstabilität der neu synthetisierten Selenohydantoine. Diese sollte sich auf verschiedene Laborumgebungen und experimentelle Bedingungen erstrecken, um die Robustheit und Reproduzierbarkeit unserer Ergebnisse im Laufe der Zeit und unter verschiedenen Umständen zu gewährleisten.

Ebenso wichtig ist es, die komplexe Natur der zellulären Redox-Homöostase und den enormen Einfluss von Organoselenmolekülen darauf zu verstehen. Die Forschung über Selen in der Redoxbiologie hat eine solide Grundlage, insbesondere im Zusammenhang mit Selenoproteinen. Die potenzielle Redoxaktivität von Selen und seine Anfälligkeit für Oxidation erfordern jedoch eine sorgfältige Untersuchung seiner Auswirkungen auf den Redoxstatus von Zellen. Auch seine Wechselwirkungen mit schwefelhaltigen Molekülen, einschließlich GSH und RSNO, müssen sorgfältig bewertet werden. Diese Überlegungen sind für ein besseres Verständnis der Rolle von Selen in der Redoxbiologie und seiner möglichen Auswirkungen auf die Gesundheit unerlässlich.

Während In-tube-Experimente zweifellos eine wichtige Forschungsgrundlage darstellen, sind In-vitro- oder *Ex-vivo*-Experimente aufgrund des Vorhandenseins verschiedener zellulärer Komponenten und Mechanismen noch komplexer. Daher haben sie einen erheblichen Einfluss auf die Ergebnisse. Darüber hinaus ist es wichtig zu erkennen, dass das Fehlen spezifischer Bindungsstellen *in vitro* oder *ex vivo* dazu führen kann, dass die tatsächliche physiologische Wirkung dieser Selenhydantoine unterschätzt wird und die beobachteten Aktivitäten reduziert werden, wie im Fall der GPx-Aktivität (*in tubo versus in vitro*).

Darüber hinaus ist die Überwachung der verschiedenen Zwischenprodukte von Seleninteraktionen von entscheidender Bedeutung. Dies beinhaltet die Verfolgung verschiedener Formen von Selen (z.B. Selenolat, Selenol, Selenon, Diselenid und Selensäure), die Beobachtung von Selenosulfid-Zwischenprodukten und die Identifizierung potenzieller (SeNO)-Spezies, die ebenfalls zur katalytischen

Freisetzung von •NO beitragen könnten. Wie bereits in einer früheren Übersicht diskutiert, sind zahlreiche spektroskopische Methoden wie UV-Vis- und NMR-Spektroskopie, Massenspektrometrie und Hochleistungsflüssigkeitschromatographie (HPLC) in der Tat wertvoll für den Nachweis verschiedener Selenspezies [79].

Darüber hinaus müssen die spezifischen experimentellen Bedingungen, einschließlich Faktoren wie Inkubationszeit und Konzentrationen, sorgfältig geprüft werden. Wie in vitro gezeigt wurde, können längere Inkubationszeiten von **5e** und **5f** zusätzliche Werte liefern, wobei beobachtet wurde, dass eine längere Wechselwirkung zwischen **5e** und GSNO den intrazellulären •NO-Pool in HuAoSMCs signifikant verändert.

Darüber hinaus könnte die Untersuchung verschiedener Konzentrationen von Selenohydantoinen zu einem besseren Verständnis der Beziehung zwischen Konzentration und Aktivität führen. Weitere Untersuchungen der Wechselwirkung mit verschiedenen Thiolen sind ebenfalls erforderlich, um die Fähigkeit dieser Selenohydantoine zur Freisetzung von •NO aus verschiedenen RSNOs zu bewerten. Darüber hinaus könnten die ex vivo beobachteten komplexen Aktivitäten die Nicht-Hill-Reaktion erklären, die auf einen Mangel an direkter Kooperativität bei der Bindung des interessierenden Moleküls an einen Rezeptor oder ein Enzym hinweist. Um die Beteiligung des sGC/cGMP-Stoffwechsels zu untersuchen, wird daher empfohlen, einen spezifischen sGC-Inhibitor wie H-[1,2,4]Oxadiazolo[4,3-a]chinoxalin-1-on (ODQ) zu verwenden, der für seine starke und selektive Hemmung der •NO-stimulierten GC-Aktivität bekannt ist. Gleichzeitig ist die Untersuchung der Modulation verschiedener Enzyme, einschließlich der Nikotinamid-Adenin-Dinukleotid-Phosphat (NADPH)-Oxidasen und der endothelialen Stickstoffmonoxid-Synthase, für die Bewertung der vasorelaxierenden Aktivität und des antioxidativen Potenzials von entscheidender Bedeutung. Insbesondere die beobachteten Aktivitäten von **5e** und **5f** an endothelfernen Aortenringen deuten darauf hin, dass sowohl endothelabhängige als auch endothelunabhängige Wege beteiligt sind. Dies wird üblicherweise durch die Verwendung von endothelunabhängigen Substanzen als Positivkontrollen erreicht (z.B. Natriumnitroprussid und Nitroglycerin), die direkt auf glatte Muskelzellen wirken und eine Vasorelaxation induzieren.

Darüber hinaus könnten In-vivo-Untersuchungen die Ergebnisse validieren. Die Untersuchung der Wirkung von Molekülen in lebenden Organismen wird ein realistischeres Bild ihrer potenziellen therapeutischen Wirkung liefern und dazu beitragen, die Lücke zwischen Laborforschung und klinischer Anwendung zu schließen. Die Untersuchung der Langzeitwirkungen ist von entscheidender Bedeutung, um ihr Potenzial als Therapeutika zu gewährleisten. Beispielsweise sind Toxizitätsstudien erforderlich, um die schädlichen Wirkungen synthetisierter Selenohydantoine in Tiermodellen zu bewerten und sowohl die akute als auch die chronische Toxizität, wie z. B. Mortalität und mögliche Anzeichen von Organschäden, zu beurteilen. Ebenso wichtig sind Studien, die untersuchen, ob eine

längere Exposition gegenüber Organoselen-Molekülen zur Entwicklung von Tumoren oder kanzerogenen Wirkungen führen kann. Ebenso wichtig ist die Bewertung der metabolischen Stabilität (z.B. Absorption, Verteilung, Stoffwechsel, Ausscheidung und Wechselwirkung mit anderen Medikamenten).

Schließlich wurden Organoselenmoleküle bereits für die Entwicklung von •NO-erzeugenden Materialien verwendet, insbesondere GPx-Mimetika, die in der Lage sind, RSNO abzubauen und so einen langfristig stabilen •NO-Pool zu erreichen. Die Entwicklung von Materialien für eine lang anhaltende Freisetzung von •NO beinhaltet beispielsweise die Verkapselung oder Immobilisierung von Organoselenmolekülen in einem geeigneten Träger, wie einem biokompatiblen Polymer oder anderen Matrizes. Techniken wie Mikroverkapselung, Einbettung in Hydrogelen oder Formulierung von Nanopartikeln können erforscht werden. Die Nanotechnologie kann zur Entwicklung von nanobasierten Formulierungen für die Verabreichung von Organoselenmolekülen (z.B. Selenohydantoinen) eingesetzt werden, was ihre Verwendung in Methoden zur verzögerten Freisetzung von •NO ermöglichen könnte. Diese Formulierungen können die Stabilität, die kontrollierte Freisetzungskinetik und die Biokompatibilität der untersuchten Moleküle verbessern und so ihre Anwendung in der kardiovaskulären Forschung erleichtern. Nanoselen und seine Anwendungen in der Nanomedizin zeigen das Potenzial für innovative Verabreichungsmethoden und eine verbesserte Bioverfügbarkeit und Sicherheit.

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Novel organoselenium catalysts: From the heart of tuna fish to antioxidant capacity linked with nitric oxide signaling in cardiovascular diseases (SeleNOx)

Cardiovascular diseases are often linked to oxidative stress and reduced bioavailability of nitric oxide (•NO), leading to disruption of vascular homeostasis. Certain small, stable organoselenium molecules display antioxidant activity similar to that of glutathione peroxidase (GPx), as well as the ability to catalyse the release of •NO from *S*-nitrosothiols (RSNOs). They can be of synthetic origin, such as ebselen, or natural, such as selenonein found in tuna. This study proposes the synthesis and evaluation *in tubo*, *in vitro* on human aortic smooth muscle cells (HuAoSMC) and *ex vivo* on isolated rat aortic rings, of new selenohydantoins inspired by selenonein. *In tubo* selenohydantoins **5e** (with $-CF_3$ substitution) and **5d** (with $-CH_3$ substitution) exhibited antioxidant, GPx-like activity and catalytic activity stimulating •NO release from *S*-nitrosoglutathione (GSNO) greater than ebselen and selenocysteamine respectively. Selenohydantoins were shown to be cytocompatible with HuAoSMC, and **5e** proved to be the best catalyst for •NO release from GSNO, significantly increasing the intracellular pool of •NO formed from nitrite ions and *S*-nitrosothiols, without affecting cellular redox balance (GPx activity and intracellular glutathione concentration). Finally, **5e** and **5f** were shown to mobilize •NO stored in the aorta in the form of *S*-nitrosothiols, inducing vasorelaxation of precontracted rat aortic rings. The results of this study suggest potential applications of synthesized selenohydantoins in the cardiovascular field, particularly in pathological conditions characterized by oxidative stress and impaired endothelial function.
key words: Selenium, Nitric oxide, *S*-nitrosation, Endothel, Vasorelaxation.

Nouveaux composés organoséléniés : du cœur du thon aux capacités antioxydantes en passant par la signalisation complexe de monoxyde d'azote (selenox)

Les maladies cardiovasculaires sont souvent liées au stress oxydant et à la réduction de la biodisponibilité du monoxyde d'azote (•NO), provoquant une perturbation de l'homéostasie vasculaire. Certains composés organoséléniés, stables et de petite taille, présentent une activité antioxydante similaire à celle de la glutathion peroxydase (GPx), ainsi qu'une capacité à catalyser la libération de •NO à partir des *S*-nitrosothiols (RSNOs). Ils peuvent être d'origine synthétique, comme l'ebselen, ou naturelle, comme la sélénonéine qui se trouve dans le thon. Cette étude propose la synthèse et évaluation *in tubo, in vitro* sur des cellules musculaires lisses aortiques humaines (HuAoSMC) et *ex vivo* sur des anneaux aortiques isolés de rat, de nouvelles sélénohydantoïnes inspirées par la sélénonéine. *In tubo* Les sélénohydantoïnes **5e** ($-CF_3$) et **5d** ($-CH_3$) ont présenté une activité GPx antioxydante et une activité catalytique stimulant la libération de •NO à partir du *S*-nitrosoglutathion (GSNO), respectivement plus importantes que celles de l'ebselen et de la sélénocystéamine. Les sélénohydantoïnes se sont montrées cytocompatibles avec des HuAoSMC et **5e** s'est révélé être le meilleur catalyseur de la libération de •NO à partir de GSNO en augmentant significativement le pool intracellulaire de •NO formé d'ions nitrites et de *S*-nitrosothiols, sans affecter l'équilibre redox cellulaire (activité GPx et concentration intracellulaire de glutathion). Enfin, **5e** et **5f** se sont révélés capables de mobiliser •NO stocké dans l'aorte sous forme de de *S*-nitrosothiols provoquant ainsi une vasorelaxation d'anneaux d'aorte de rat précontractés. Les résultats de cette étude suggèrent de potentielles applications de sélénohydantoïnes synthétisées dans le domaine cardiovasculaire, en particulier dans des conditions pathologiques caractérisées par un stress oxydant et une dégradation de la fonction endothéliale.
Mots-clefs : Sélénium, Monoxyde d'azote, *S*-nitrosation, Vasorelaxation, Endothélium

Neuartige Organoselenium-Katalysatoren: Vom Thunfischherz zur antioxidativen Kapazität in Verbindung mit Stickoxid-Signalen bei Herz-Kreislauf-Erkrankungen (SeleNOx)

Kardiovaskuläre Erkrankungen sind mit oxidativem Stress und einer verminderten Bioverfügbarkeit von Stickstoffmonoxid (•NO) verbunden, was die vaskuläre Homöostase stört. Diese Studie untersucht die potentielle Modulation der vaskulären Funktion durch neue Organoselenmoleküle mit potentieller GPx-ähnlicher Aktivität, antioxidativer Aktivität und •NO-Freisetzung aus *S*-Nitrosothiolen (RSNOs). Diese Moleküle können synthetisch (wie Ebselen) oder natürlich (wie Selenonein in Thunfisch) sein. Inspiriert von Selenonein wurden neue Selenohydantoine synthetisiert und *in tubo*, *in vitro* an humanen glatten Muskelzellen der Aorta (HuAoSMC) und *ex vivo* an Ratten-Aortenringen evaluiert. Die Experimente *in tubo* zeigten eine interessante GPx-ähnliche Aktivität in der Nähe von Ebselen, insbesondere der Derivate **5e** ($-CF_3$) und **5d** ($-CH_3$). Selenohydantoine zeigten hohe antioxidative Aktivitäten und katalytische Eigenschaften, die die Freisetzung von •NO aus RSNOs stimulierten. Z.B. übertrafen die Derivate **5e** und **5f** Selenocystamin. *In vitro*- und *ex vivo*-Experimente zeigten die Zytokompatibilität der Selenohydantoine, wobei das Derivat **5e** die Protein-*S*-Nitrosation in HuAoSMC induzieren konnte, was den intrazellulären •NO-Pool signifikant erhöhte, ohne das zelluläre Redoxgleichgewicht (GPx-Aktivität und Glutathionkonzentration) zu beeinflussen. Wenn es mit *S*-Nitrosoglutathion (GSNO) coinkubiert wurde, ermöglichte es eine potentiell positive kardiovaskuläre Wirkung. Tatsächlich zeigten die Derivate **5e** und **5f** die Fähigkeit zur Vasorelaxation an vorverengten Ratten-Aortenringen und die Fähigkeit, GSNO-induzierte •NO-Reserven in der Aorta für die Vasorelaxation zu mobilisieren.
Schlüsselwörter: Selen, Stickstoffmonoxid, *S*-Nitrosierung, Endothel, Vasorelaxation.