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Chapter 1**Engineering Peptidyl and Protein
Glutathione Peroxidase Mimics****1.1 Introduction**

Peptides are central in terms of size in the molecular continuum of pharmaceutical tools ranging from small molecules to proteins. However, their chemical and biochemical properties are very distinct from both classes of pharmaceuticals. Until the end of the 20th century, examples of peptides on the market were very scarce, with insulin as the blockbuster [1,2]. This can be explained by the main drawbacks of peptides such as their poor stability in biological media, leading to suboptimal pharmacokinetics and their poor membrane permeability. However, since 2000, more and more peptides enter clinical development and the number of approved peptides increases gradually. The proteolytic instability and rapid clearance of peptides are nowadays seen as key advantages since peptides do not accumulate in tissues, are not metabolized in the liver avoiding drug-drug interactions and non-anticipated toxicology, contrarily to what happens with small molecules. In addition, their production is much easier than proteins production and they present a lower immunogenicity. The majority of peptidyl pharmaceuticals are produced by chemical methods, using mainly solid phase peptide synthesis, but some of them are produced using recombinant techniques. They have consequently benefited from the improvements in chemical and biological technologies for their production or delivery. Nowadays, approved peptides are used as drugs targeting proteins (mainly G-protein coupled receptors, GPCRs or other cell surface receptors) exploiting their recognition properties or mean to deliver a cytotoxic payload or imaging agent. They are thus considered as “stoichiometric” drugs since one molecule reacts with one target (a protein for instance). On the contrary, a new class of drugs called

catalytic drugs is emerging with promising applications [3,4]. Drugs based on catalysis afford potentially great advantages over stoichiometric drugs as they may allow decreasing the administered doses and thus potential side effects. Catalytic drugs are mainly metal complexes inspired from metalloenzymes such as oxidoreductases, lyases, transferases, hydrolases, ligases and isomerases that catalyze very efficiently elemental reactions. Recently, five major groups of bioinspired catalytic drugs have been explored: photosensitizers used in photodynamic therapy to generate singlet oxygen [5], catalysts of transfer hydrogenation for the regeneration of NADH and NAD⁺ [6], catalysts of peptide bonds or phospho-diester bond hydrolysis [3,7] used for hydrolytic cleavage of amyloids [8–10], HIV1 or hepatitis C RNA [12,13], thiol oxidants and in particular glutathione oxidants [14] and antioxidant enzymes mimics. The development of superoxide dismutase (SOD) [15–21], catalase (CAT) [21–24] and glutathione peroxidase (GPx) [23,25–29] mimics have attracted much attention as these enzymes play a central role in the redox homeostasis by controlling the levels of superoxide (SOD) and hydroperoxides (CAT and GPx) known as reactive oxygen species (ROS). SODs catalyze the dismutation of superoxide (O₂^{•-}) into O₂ and H₂O₂, CAT the dismutation of H₂O₂ into O₂ and H₂O and GPx the reduction of ROOH into ROH. Among antioxidant enzyme mimics developed as catalytic drugs, several of them are peptides or involve a peptide as ligand of the metal cation bearing the redox properties essential for the catalysis. For example, peptidyl copper [30–34] or manganese [35,36] complexes have been developed as SOD mimics. However, no peptidyl metal complex mimicking CAT has been described so far. Concerning GPx, several strategies have been undertaken to develop selenium containing GPx mimics and this will be the focus of this book chapter.

Glutathione peroxidase GPx is a family of enzymes that catalyze the reduction of hydroperoxides (ROOH) including hydrogen peroxide (H₂O₂) into their corresponding alcohols (ROH or H₂O for H₂O₂). Concomitantly thiols are oxidized into disulfide, with glutathione (GSH) as the mostly involved reductant [37]. Some GPx are selenoenzymes, meaning that they involve at their catalytic site a selenocysteine (Sec, U), often called the 21st amino acid [38]. Selenocysteine is an analog of the

cysteine, the selenol group replacing the thiol group. Selenocysteine has a lower pKa (5.24–5.63) than cysteine's one (8.25), which suggests at physiological pH the Sec residue will be present predominantly in the reactive selenolate form [39]. In addition, it is a stronger nucleophile, a better leaving group and diselenides are more prone to nucleophilic attack than disulfides. Finally, Sec has also a lower reduction potential than Cys. Altogether, these properties make it very efficacious in proteins possessing antioxidant activities. The incorporation of the selenocysteine in GPx is not a post translational modification but this amino acid is encoded by a UGA codon in messenger RNA, which is usually a STOP codon. In certain circumstances that will be not discussed here, this STOP codon leads to the incorporation of the selenocysteine [40].

Primarily, because of their ability to reduce hydroperoxides into less reactive species, GPx were thought to detoxify organisms by preventing the formation of highly reactive radicals resulting from their degradation. GPx1 Knockout Mice ((-/-), mice with no GPx1) submitted to an oxidative stress induced by paraquat did not survive even if they were supplemented by selenium whereas selenium-supplemented wild type mice survived [41]. This clearly demonstrates that the mammalian GPx1 plays a crucial role as antioxidant defense. However, later on, it has been shown that hydroperoxides are involved in various signaling pathways [42]. Consequently, the physiologic role of GPx is not restricted to antioxidant defense but to many others physiologic processes involving redox reactions. Brigelius-Flohé et Maiorino have recently reviewed the mammalian GPx functions thus giving an overview of their key regulating roles of multiple pathways that respond to hydroperoxides including H₂O₂ [43].

1.2 Mammalian GPx

1.2.1 Mammalian isoforms: localization and substrate specificities

Mammalian GPx family is composed of eight different isoforms GPx1-8 (Table 1). Among these isoforms, GPx1-4 and GPx6 contain a selenocysteine and are called SecGPx whereas GPx5 and GPx7-8 possess a cysteine at their catalytic site (CysGPx) [44]. In addition to the composition of their active sites, these isoforms also differ from their location and thus from their substrates scope. GPx1 is found in the cytosol and reacts with soluble hydroperoxides such as H₂O₂, *t*-butyl hydroperoxide and hydroperoxide of simple lipids. GPx2, possessing a closely related structure, may possess the same substrates but has not been purified so far, thus preventing the study of its substrates scope. As GPx2 is mainly expressed in intestinal epithelium, it was suggested that its role was to reduce the toxicity of ingested lipid hydroperoxides [45]. GPx3 is also very similar to GPx1 but lacks an endoplasmic reticulum (ER) retention signal, which may be responsible for its release into the plasma where it reacts with soluble hydroperoxides as well. Three different isoforms of GPx4 have been described. They are located in the cytosol, mitochondria, nucleus or at the plasma membrane [46]. Very important features of GPx4 are that it is a monomeric protein, contrarily to the others Sec containing GPx and lacks the GSH binding sequence. These specificities provide unique properties to GPx4: it is able to reduce, beside small hydroperoxides, hydroperoxides of complex lipids and of cholesterol and it can accommodate thiol proteins as reductant acting as thiol peroxidase. GPx5 is mainly secreted in the epididymis and is closely related to GPx3. GPx6 is homologous to GPx3 but very little is known about its reactivity. It is mainly located in the olfactory epithelium. GPx7 is the cysteine-containing counterpart of the GPx4 and is mainly located in the lumen of the ER. To finish, GPx8 is a membrane protein of the ER and the later detected member of the GPx family.

Since little is known about the catalytic cycle of the mammalian Cys-containing GPx, only SecGPx that have been more deeply investigated will be discussed in the following.

Table 1. Structural features, proven substrates and 2nd order kinetics constants of mammalian GPx. Adapted from references [43] and [47].

GPx type	Peroxidatic residue	Homo-tetramer	GSH binding domain ^[a]	Reducing substrate	Location	Oxidizing substrate	kinetic constants (M ⁻¹ s ⁻¹)
GPx1	Sec	Yes	R57, R103, R184, R185, K91'	GSH	cytoplasm	H ₂ O ₂ , ROOH, soluble LOOH, ONOO ⁻	k_{+1} : 4.1 10 ⁷ [b] k'_{+2} : 2.3 10 ⁵ [b]
GPx2	Sec	Yes	R57, R103, R184 conserved	n.d.	gastro intestinal epithelium	n.d.	
GPx3	Sec	Yes	R103, R185 conserved	GSH, low rate with Trx and Grx	plasma	H ₂ O ₂ , ROOH, soluble LOOH, PLOOH	k_{+1} : 4.0 10 ⁷ [b] k'_{+2} : 7.9 10 ⁴ [b]
GPx4	Sec	No	No	GSH, DTT, mercaptoethanol. protein thiols	cytosol, mitochondria, nucleus	H ₂ O ₂ , ROOH, PLOOH, LOOH, ChOOH, ONOO ⁻ , CEOOH	k_{+1} : 1.5 10 ⁷ [c] k'_{+2} : 5.7 10 ⁴ [c]
GPx5	Cys	Yes	R103, R185 conserved	n.d.	epididymis	n.d.	
GPx6	Sec	Yes	n.d.	n.d.	olfactory epithelium	n.d.	
GPx7	Cys	No	n.d.	GSH, PDI?	ER lumen	H ₂ O ₂	
GPx8	Cys	No	n.d.	GSH, PDI?	ER membrane	H ₂ O ₂	k_{+1} ^a 95 ^[a] k'_{+2} n.d.

^[a] Bovine GPx numbering. ^[b] with H₂O₂. ^[c] with phosphatidylcholine hydroperoxide

ChOOH, cholesterol hydroperoxide; CEOOH cholesterol ester hydroperoxide; DTT, dithiotreitol; Grx, glutaredoxin; LOOH, lipid (fatty acid) hydroperoxide; PLOOH, phospholipid hydroperoxide of different classes; ROOH, small synthetic hydroperoxides (e.g. cumene hydroperoxide and tert-butyl-hydroperoxide); Trx, thioredoxin; PDI, protein disulfide isomerase; n.d. not determined so far.

1.2.2 Catalytic activity of mammalian SecGPx

The catalytic cycle of SecGPx comprises three steps (Figure 1a) [43,48]. The first step is the oxidation of the selenol under its selenolate (GPxSe⁻) form of the GPxSeH Sec residue with hydroperoxide. This Sec residue and its Cys counterpart in Cys-containing GPx (CysGPx) are thus commonly called the peroxidatic residues. This oxidation leads presumably to the formation of a highly reactive selenenic acid (GPxSeOH) that has not been evidenced so far. In a second step, the selenenic acid is reduced with GSH to form water and a selenenylsulfide (GPxSeSG), which is then reduced in a final step by another GSH to produce GSSG in one hand and to recover the GPxSeH in the other hand. The overall cycle is thus a three-substrate reaction following a non-sequential mechanism that in kinetics term is referred as a ping-pong mechanism [37]. The first step of the catalytic cycle, corresponding to the reaction with the hydroperoxide, is also called the peroxidatic part and the two last steps belong to the reductive part of the cycle as it included two successive reductions. From this simplified two-parts mechanism, two apparent second order rate constants have been defined (k_{+1} and k'_{+2}) where k_{+1} describes the oxidation by hydroperoxide and k'_{+2} the sum of the rate constants for the two reductions. The overall rate is thus $v = [\text{GPx}]_0(k_{+1}[\text{ROOH}] + k'_{+2}[\text{GSH}])$. The relevant steady-state equation for the enzymatic peroxidase reaction following a ping pong mechanism is thus written:

$$\frac{v_0}{[\text{GPx}]_0} = \frac{k_{\max}[\text{GSH}][\text{ROOH}]}{K_{\text{m,ROOH}}[\text{GSH}] + K_{\text{m,GSH}}[\text{ROOH}] + [\text{GSH}][\text{ROOH}]}$$

where k_{\max} is a pseudo-first-order rate constant and $K_{\text{m,ROOH}}$ and $K_{\text{m,GSH}}$ are the Michaelis constants for the peroxide and thiol, respectively [49].

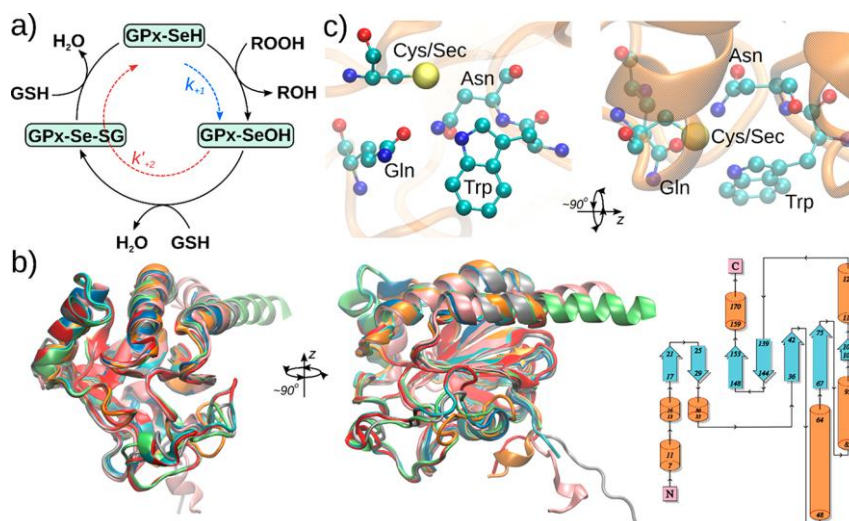
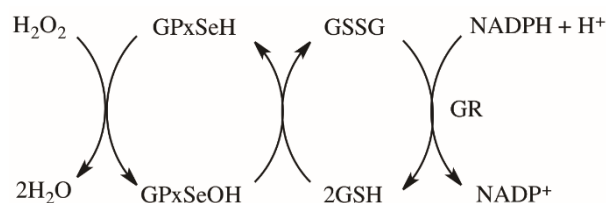


Figure 1. Catalytic cycle and structural features of GPxs. (a) GPxs “canonical” catalytic cycle scheme. (b) Structure superimposition of several human GPx: GPx1, cyan (PDB 2F8A); GPx2, red (PDB 2HE3); GPx3, green (PDB 2R37); GPx4, orange (PDB 2OBI); GPx5, gray (PDB 2I3Y); GPx7, blue (PDB 2P31); GPx8, pink (PDB 3CYN). A topological diagram of GPx4 as a representative family member is also shown. (c) Catalytic tetrad of GPx: besides the reactive Cys/Sec residue, the conserved critical Gln, Trp, and Asn residues are highlighted. Copyright 2019, American Chemical Society [47].

To determine the kinetics constants, stopped flow techniques were indeed used to measure H_2O_2 concentrations.[49] An assay initially described by Wendel [50] and modified by Wilson *et al.* [51] has then been developed to measure in a more routine fashion the kinetics using a standard UV-vis spectrometer. This assay involves glutathione reductase (GR) with nicotinamide adenine dinucleotide phosphate (NADPH) to regenerate GSH from GSSG. In this situation, the GSH concentration remains essentially constant, leading to pseudo-zero order kinetics, and the decrease in NADPH can be continuously measured spectrophotometrically at 366 nm (scheme 1). The decrease in NADPH concentration is directly related to the GPx activity. Nevertheless, the values obtained are still dependent on the GSH concentration during the cycle.



Scheme 1. Catalytic cycle of the GPx activity assay involving glutathione reductase (GR) and NADPH. Scheme adapted from reference [52]. The resulting chemical equation is $\text{H}^+ + \text{NADPH} + \text{H}_2\text{O}_2 \rightarrow \text{NADP}^+ + 2\text{H}_2\text{O}$.

The catalytic site of GPx has first been described as a triad involving the selenocysteine (Sec), glutamine (Gln) and tryptophan (Trp) [48]. It has been proposed that hydrogen-bonds between the amido and imino groups of the Gln and Trp, respectively, with the Sec residue activate the selenol to facilitate the nucleophilic attack on the hydroperoxide [53]. Following this attack, the selenolate is readily oxidized to the selenenic acid derivative. Later on, it has been shown by the evaluation of different GPx mutants of *Drosophila melanogaster* GPx (DmGPx), described as a paradigm of the GPx family, that an asparagine (Asn) plays also a pivotal role in the catalytic activity (Figure 1b and c) [54]. It has been emphasized that the contribution of the asparagine amido group for activation of the selenol (and the thiol for CysGPx) through hydrogen bonding is even more important than the glutamine and tryptophan contribution.

This tetrad is identical in all mammalian GPx active site, except in GPx8 where the glutamine is replaced by a serine [55]. Another key feature of the GPx is their ability to interact or to direct the reductant to favor the reduction of the selenenic acid. The X-ray structure of the bovine GPx1 has shown that the catalytic site is surrounded by four arginines and one lysine that may interact with the negatively charged carboxylate groups of GSH [48]. Further investigations by molecular modelling have supported the importance of these residues for GSH binding [56].

Tremendous efforts have been devoted to the generation of GPx mimics to (i) better understand their catalytic activity and (ii) to develop

efficient molecules able to supplement GPx when these enzymes are inactive or overwhelmed. Many small molecules containing selenium have been developed to mimic GPx with ebselen as the gold standard compound [57,58]. These organoselenium compounds have been extensively reviewed [25,28,29,59,60] and are not in the scope of this book chapter, which focuses on mimics involving proteins and peptides.

1.3 GPx mimics

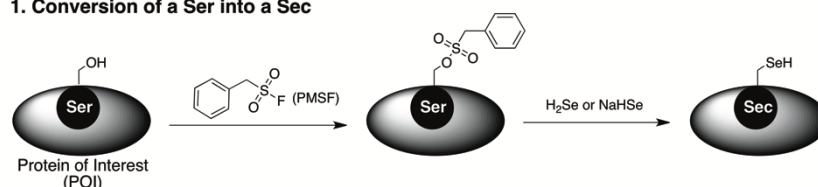
1.3.1 Protein mimics of GPx

1.3.1.1 Engineering GPx by incorporating a selenocysteine into a protein scaffold

In a pioneer work, Hilvert and coworkers have converted a serine of the active site of the serine protease subtilisin protein into a selenocysteine [61]. For this purpose they have used two successive chemical reactions: a reaction with phenylmethanesulfonyl fluoride (PMSF) to generate a good leaving group followed by a nucleophilic substitution using hydrogen selenide (H_2Se) as nucleophile (Figure 2). They have then investigated in details the GPx activity of this newly created selenosubtilisin (Table 2) [62–64]. In a similar way, Liu *et al.* have converted a trypsin into a selenotrypsin that exhibited GPx-like activity (Table 2) [65]. Few years later, Branlant *et al.* have converted the cysteine of the phosphorylating glyceraldehyde 3-phosphate dehydrogenase (GAPDH) active site into a selenocysteine [66]. This has been achieved by using the strategy developed by Böck *et al.* [67]. In brief, after having demonstrated that the cysteine can be replaced by the selenocysteine into the cysteine-specific tRNA by the cysteinyl-tRNA ligase, they produce the protein in bacteria under cysteine deprivation conditions and selenocysteine supplementation. This resulted in the incorporation of selenocysteine instead of cysteine. The produced selenoprotein (SeGAPDH) has shown similar second order catalytic activity as selenosubtilisin but with optimum values for different hydroperoxides (Table 2). These modified proteins were all found much

less efficacious than the native enzyme most probably because the active site of these mimics is buried inside the protein matrix whereas it is at the surface of native GPx enzyme and they do not possess a GSH binding site.

1. Conversion of a Ser into a Sec



2. Selenocysteine mediated native chemical ligation (SeNCL)

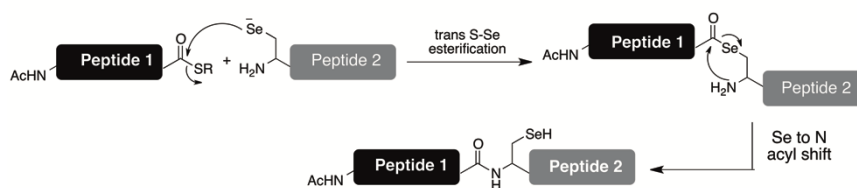


Figure 2. Chemical strategies to incorporate a selenocysteine into a protein.

1.3.1.2 Introducing GSH binding properties into GPx mimics

a) Catalytic antibodies

In order to confer GSH binding properties to such GPx mimics, Luo et al. have developed a selenium containing monoclonal antibody with catalytic activity (abzyme) [68]. For this purpose, they have first produced a monoclonal antibody that selectively binds GSH with high affinity. Then, this antibody has been chemically modified following the 2-step procedure described by Hilvert [61] to substitute the hydroxyl group of serine residues of the monoclonal antibody L chain by selenol. In this construct (Se-4A4), both the GSH binding domain and the active site were located on the variable part of the monoclonal antibody providing spatial proximity that conferred good catalytic activity (Table 2) [69]. A similarly prepared abzyme 4mG3 [70] has been investigated for its antioxidant properties on isolated myocardial mitochondria. It has been shown that 4mG3 was able to protect mitochondria submitted to a

flow of reactive oxygen species produced by the enzymatic system xanthine oxidase/hypoxanthine [71]. Few years later, these abzymes were improved by modifying the preparation of the monoclonal antibodies and they ended up with GPx mimics whose second order constant were only 2 orders of magnitude lower than native GPx one. In order to produce such mimics in high quantities, the variable region of the best monoclonal antibody (2F3) has been cloned and expressed as a single-chain fragment in different bacterial strains and then chemically modified to incorporate the reactive selenol (Se-2F3-scFv) [72]. Very interestingly, this new construct keeps the ability to reduce H_2O_2 and showed a very good activity ($2840 U \mu mol^{-1}$, which is 49% of that of the rabbit liver GPx : $5780 U \mu mol^{-1}$) [73]. The antioxidant properties of Se-2F3-scFv were then investigated on a damaged-mitochondria model system and it showed capacity to decrease mitochondrial swelling, to inhibit lipid peroxidation and to maintain cytochrome c oxidase activity [73]. In addition, the antioxidant properties of this single chain Fv on rat skin epidermal cells exposed to damaging UV-B were demonstrated: Se-2F3-scFv inhibited lipid peroxidation, preserved membrane integrity and increased cell viability [27].

b) Engineering proteins that intrinsically possess affinity for glutathione

With the same goal of introducing GSH binding properties to improve the efficacy of the GPx mimics, Luo *et al.* have converted a glutathione S-transferase (GST), that is modular, stable and easy to synthesize and more importantly that already possesses a GSH binding motif, into a GPx mimic. They have chemically converted the serine of the GST into a selenocysteine leading to a semi-synthetic GPx mimic called Se-rGST T2-2 with both high thiol selectivity and high catalytic efficiency (Table 2) [74]. However, using a chemical modification of serine residues lacks selectivity since several serines are present in GST.

Table 2. Selected engineered GPx mimics and their kinetics parameters.

	Sec incorporation	ROOH	k_{cat} (app) min^{-1}	K_m , ROOH mM	K_m , thiol μM	k_{cat}/K_m , ROOH $10^6 \text{ M}^{-1} \text{ min}^{-1}$	References
GPx1 (rabbit liver) ^[a]		H ₂ O ₂	47200	0.0044		1000	[49,75,76]
Selenosubtilisin ^[b]		<i>t</i> -BuOOH	2100 ± 200	530 ± 60	160 ± 20 (ArSH)	0.0039 ± 0.0002	[64]
	Chemical conversion of Ser	CuOOH				0.018 ± 0.001	[64]
		H ₂ O ₂	> 8000	> 0.3	> 400 (ArSH)	0.0137 ± 0.0008	[64]
Selenotrypsin ^[c]	Chemical conversion of Ser	H ₂ O ₂	124 ± 4	1.25 ± 0.11	1310 ± 160	0.099	[65]
SeGAPDH ^[d]		<i>t</i> -BuOOH	228	430	60 (ArSH)	0.00053	[66]
	Genetic incorporation	CuOOH	14	2	140 (ArSH)	0.007	[66]
		H ₂ O ₂	N.D.	N.D.	N.D.	0.023 ± 0.001	[66]
Se-4A4 ^[a]	Chemical conversion of Ser	H ₂ O ₂	619.5	0.09	270	6.86	[68,69]
Se-2F3 ^[a]	Chemical conversion of Ser	H ₂ O ₂	25400	1.19	20.4	21.1	[75]
Se-rGST T2-2 ^[c]	Chemical conversion of Ser	H ₂ O ₂	20000 ± 200	0.11 ± 0.02		180 ± 12	[74]
Se-GSTLu1-1 ^[c]	Genetic incorporation	H ₂ O ₂	2100 ± 30	0.21 ± 0.01	170	9.90 ± 0.28	[77]
Selenogluta redoxin ^[e]	Sec Native chemical ligation	<i>t</i> -BuOOH	0.042				[78]
Biomprinted egg albumin ^[f]	Chemical conversion of Ser	H ₂ O ₂	785				[79]
Bioimprinted selenosubtilisin ^[g]	Chemical conversion of Ser	H ₂ O ₂	1170 ± 50	0.62 ± 0.08	300 ± 20	1.89 ± 0.05	[80]

Reaction conditions: a) [GSH] = 1 mM, [H₂O₂] = 0.5 mM, [NADPH] = 0.25 mM, [EDTA] = 0.1 mM, [GR] = 1 U and [NaN₃] = 1 mM in phosphate buffer (50 mM, pH 7) at 37°C. Reaction performed with the Wendel method.[50] b) [EDTA] = 1 mM, [CaCl₂] = 10 mM in buffer (citric acid 33 mM, HEPES 33 mM, MES 33 mM, pH 5.5) and 10% DMF at 25°C°. ArSH: 3-carboxy-4-nitrobenzenethiol. Reaction followed by stopped-flow spectroscopy. c) [GSH] = 0.25 mM, [H₂O₂] = 0.5 mM, [NADPH] = 0.21 mM, [EDTA] = 1 mM, [GR] = 1 U, [NaN₃] = 1 mM in phosphate buffer (50 mM, pH 7) at 37°C. Reaction performed with the Wendel method.[50] d) [ArSH] = 0.2 mM, [ROOH] = 10 mM, [EDTA] = 0.2 mM, ionic strength: 0.15 M in MES (100 mM, pH 5) at 25°C. ArSH: 4-nitrobenzenethiol. e) [GSH] = 0.01-21 mM, [*t*-BuOOH] = 0.1 mM, [NADPH] = 0.21 mM, [EDTA] = 1 mM, [GR] = 5 U mL⁻¹, [NaCl] = 100 mM in phosphate buffer (50 mM, pH 7) at 25°C.[51] f) [GSH] = 1 mM, [H₂O₂] = 0.5 mM, [NADPH] = 0.25 mM, [GR] = 1 U in phosphate buffer (50 mM, pH 7) at 25°C. Reaction performed with the Wendel method.[50] g) [GSH] = 1 mM, [H₂O₂] = 0.5 mM, [NADPH] = 0.25 mM, [EDTA] = 1 mM, [GR] = 1 U, [CaCl₂] = 10 mM in PIPES buffer (50 mM, pH 7) at 37°C. Reaction performed with the Wilson method.[51]

To avoid that, they have used a GST mutant of the Australian sheep blowfly (*Lucilia cuprina*) LuGST1-1 where, first, the serine 9 (Ser9) of the catalytic site has been replaced by a cysteine and then the two cysteines (Cys86 and Cys200) were replaced by serine [77]. The authors argued that since these cysteines are apart from the active site and are not involved in a disulfide bridge, these substitutions did not impact the protein structure and catalytic activity. The LuGST1-1 mutant was converted into a selenoprotein (SeLuGST1-1) using the already described Böck et al. strategy [67] that relies on the fact that selenocysteine is charged on the cysteine-specific tRNA when the growth medium is depleted in cysteine and enriched in selenocysteine. SeLuGST1-1 appeared to be less efficient than Se-rGST T2-2 most probably because only a single residue has been converted into a selenocysteine in SeLuGST1-1 whereas several could have been introduced using the chemical strategy (Table 2). Hilvert *et al.* have also converted a mutant of *E. coli* glutaredoxin 1 (Grx1) into a GPx mimic using a three-fragment ligation strategy involving one conventional cysteine-based and one selenocysteine-based native chemical ligation step (SecNCL) (Figure 2) [78]. This semi synthetic enzyme selenoglutaredoxin exhibited a low GPx-like activity, most probably because its monomeric structure does not prevent the formation of a diselenide dimer that competes with the formation of the required selenenylsulfide intermediate (Table 2).

c) Bio imprinted proteins

An alternative strategy that has been developed to produce protein-based GPx mimic with a GSH binding domain relied on a combination of bioimprinting and chemical modification. This method requires a protein matrix and an imprinting molecule to generate a binding site. In brief, the bioimprinting relies on four steps: (i) protein unfolding, (ii) addition of the imprinting molecule to enable interactions between the molecule and the protein, (iii) crosslinking of the protein to block the conformation of the molecule-protein adduct, (iv) removing of the imprinting molecule. Finally, in the case of GPx mimics, serine residues were chemically converted into selenocysteines. Luo et al. used egg albumin as the protein matrix and a modified GSH as imprinting molecule (N,S-bis(2,4-

dinitrophenyl)glutathione, GSH-2DNP). Following this strategy, they produced an egg albumin imprinted GPx that was only seven times less active than the native GPx from rabbit liver (817 and 5780 U μmol^{-1} for the bioimprinted protein and native GPx respectively) [79]. To improve catalytic efficiency, another imprinted protein mimicking GPx has been produced starting from Hilvert's selenosubtilisin. The GSH binding site was created through the crosslinking of the partially denatured GSH-selenosubtilisin adduct using glutaraldehyde [80]. As expected, this bioimprinted selenosubtilisin reacted with GSH whereas selenosubtilisin did not and with second order kinetics that are 2 orders of magnitude higher than selenosubtilisin.

1.3.2 Peptides as GPx mimics

As highlighted earlier, only few of these semi synthetic enzymes have been used in more biologically relevant conditions. As native proteins, semi synthetic enzymes have poor pharmaceutical potentials since they have large molecular weight, can exhibit immunogenicity and membrane permeability issues. Consequently, smaller peptidyl mimics have been developed to overcome these shortcomings. They all include a selenocysteine residue as the peroxidatic residue which is oxidized into a diselenide to form dimers that are more stable than the monomers.

1.3.2.1 Primary amino acid sequence models

Yoshida *et al.* have studied diselenide dimers of tri- and pentapeptides composed of the amino acids involved in the native primary sequence (residues 48 to 50 or 47 to 51) of the human GPx [52]. These peptides have been synthesized on solid support using a Fmoc strategy (Table 3, entries 1 and 2). Interestingly, they have shown that $(\text{H}_2\text{N-Leu-Sec-Gly OH})_2$ and $(\text{H}_2\text{N-Ser-Leu-Sec-Gly-Thr-OH})_2$ exhibited a GPx-like activity in presence of GSH and that the longer peptide $(\text{H}_2\text{N-Ser-Leu-Sec-Gly-Thr-OH})_2$ was less active than the shorter one $(\text{H}_2\text{N-Leu-Sec-Gly OH})_2$. This thus suggested that the primary amino acid sequence around the GPx peroxidatic residue does not play a pivotal role for the antioxidant activity [26]. This was confirmed by Wehrle *et al.* that

have studied an extended primary sequence mimic of the human GPx (from residue 49 to 58) [81]. This peptide (Table 3, entry 3) exhibited a poor catalytic activity. However, by substituting the selenocysteine by α -methyl selenocysteine, they ended up with a more stable and active peptide (Table 3, entry 4). However, the initial rate was still low highlighting the fact that mimicking the primary sequence around the Sec residue is not a valuable strategy.

1.3.2.2 *Reproducing the catalytic triad using model peptides*

Through a Monte Carlo simulation, Iwaoka *et al.* have determined amino acid sequences involving a selenocysteine that could be interesting to reproduce the catalytic triad of GPx [82]. A peptide possessing a GQAUAWG (Table 3, entry 5) sequence has emerged and has been studied as a diselenide dimer (GQAUAWG)₂ together with several variants lacking the glutamine or/and the tryptophan residues (Table 3, entries 5-8). All selenopeptides showed GPx-like activity higher than primary sequence mimics and the results supported a cooperative effect of the Gln and Trp residues on the activity. This highlights the importance of the catalytic triad. In addition, it has been proposed that Gln and Trp were involved in NH...Se hydrogen bonds in the selenolate form and that in the SeOH and SeSG forms, the amide of Gln was pushed away and the indole ring of the Trp was involved in a CH...Se hydrogen bond.

Table 3. Peptidyl SecGPx mimics and their GPx activity. The peroxidatic Sec residue is indicated in red, the residues involved in the catalytic tetrad in bold and residues that may confer GSH binding are in italic.

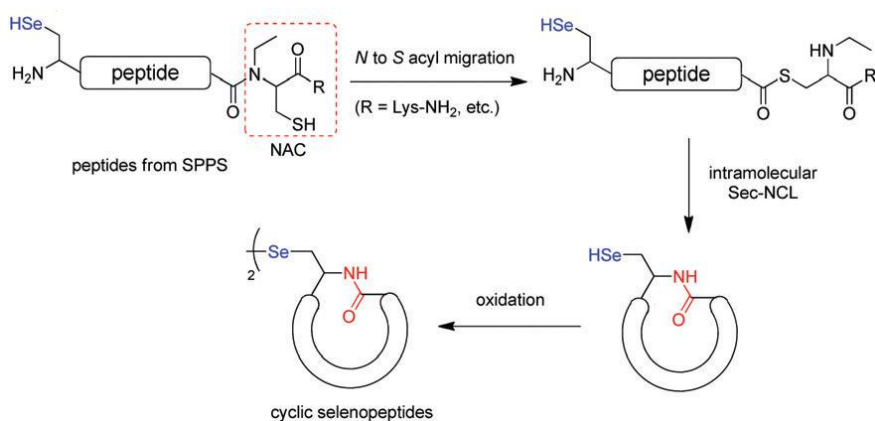
	Entry	Peptide sequence	v_0 $\mu\text{M min}^{-1}$	GPx activity $\text{U } \mu\text{mol}^{-1}$	$k_{\text{cat}}/K_{\text{m,H}_2\text{O}_2}$ $\text{M}^{-1} \text{min}^{-1}$	References
Primary sequence mimics	1	(H-Leu- Sec -Gly-OH) ₂	44 ± 6 ^[a]			[52]
	2	(H-Ser-Leu- Sec -Gly-Thr-OH) ₂	39 ± 3 ^[a]			[52]
	3	(H- Sec -Gly-Thr-Thr-Val-Arg-Asp-Tyr-Thr-Gln-OH) ₂	18.0 ± 0.4 ^[b]			[81]
	4	(H-(<i>α</i> Me) Sec -Gly-Thr-Thr-Val-Arg-Asp-Tyr-Thr-Gln-OH) ₂	23.5 ± 1.9 ^[b]			[81]
Catalytic triad mimics	5	(H-Gly- Gln -Ala- Sec -Ala- Trp -Gly-OH) ₂	617 ± 50 ^[a]			[82]
	6	(H-Gly- Gln -Ala- Sec -Ala-Ala-Gly-OH) ₂	550 ± 83 ^[a]			[82]
	7	(H-Gly-Ala-Ala- Sec -Ala- Trp -Gly-OH) ₂	317 ± 17 ^[a]			[82]
	8	(H-Gly-Ala-Ala- Sec -Ala-Ala-Gly-OH) ₂	383 ± 33 ^[a]			[82]
Catalytic tetrad mimics	9	(H-Lys- Gln -Gly- Sec -Gly- Trp -Gly- Asn -OH) ₂	1167 ± 83 ^[a]			[83]
	10	(H-Arg- Gln -Ala- Sec -Ala- Trp - Asn -Gly-OH) ₂	783 ± 50 ^[a]			[83]
	11	(H-Arg- Gln -Pro- Sec -Pro- Trp - Asn -Gly-OH) ₂	833 ± 50 ^[a]			[83]
	12	(c-Lys- Gln -Gly- Sec -Gly- Trp -Gly- Asn -) ₂	1483 ± 133 ^[a]			[83]
	13	(c-Lys- Gln -Gly- Sec -Gly-Ala-Gly- Asn -) ₂	1400 ± 67 ^[a]			[83]
	14	(c-Lys- Gln -Gly- Sec -Gly- Trp -Gly-Ala-) ₂	1200 ± 50 ^[a]			[83]
	15	(c-Lys-Ala-Gly- Sec -Gly- Trp -Gly- Asn -) ₂	633 ± 67 ^[a]			[83]
GSH mimic	16	(H- <i>γ</i> -Glu- Sec -Gly-OH) ₂	> 210 ^[a]			[52]
Mimic with GSH binding	17	(H-Arg-Gly- Sec -Arg-Asn-OH) ₂		10.9	8.54 10 ⁴ ^[c]	[84]
	18	(H-Arg- Sec -Gly-Arg-Asn-Ala-Gln-OH) ₂		13 ^[d]		[85]
15SeP ₁	19	(H-Gly-Pro-Pro-Pro-Arg-His-Asn-Val-Tyr-Gly-Arg-Pro-Arg-Ala- Sec -OH) ₂		190	3.07 10 ⁷ ^[e]	[76]
15SeP	20	(H-Trp-Pro-Phe-Leu-Arg-His-Asn-Val-Tyr-Gly-Arg-Pro-Arg-Ala- Sec -OH) ₂		260	2.42 10 ⁸ ^[e]	[76]

GPx (rabbit liver)	21	5780	$6.60 \cdot 10^{21}$	[86]
			10^9	34

Reaction conditions: a) [GSH] = 4 mM, [H₂O₂] = 0.25 mM, [NADPH] = 0.3 mM, [GR] = 4 units mL⁻¹ and [catalyst] = 50 μM in phosphate buffer (pH 7.4) at room temperature. b) [GSH] = 0.1 mM, [NADPH] = 0.2 mM, [EDTA] = 1 mM, [GR] = 4 unit/mL, [H₂O₂] = 0.2 mM, [catalyst] = 10 μM in potassium phosphate buffer (100 mM, pH 8) at 25°C. c) [GSH] = 0.5-3 mM, [H₂O₂] = 0.5-2 mM, [NADPH] = 0.25 mM, [EDTA] = 1 mM, [GR] = 1 unit, and [catalyst] = 5 μM in potassium phosphate buffer (50 mM, pH 7.4) at 37°C. d) [GSH] = 1 mM, [H₂O₂] = 0.5 mM, [NADPH] = 0.25 mM, [EDTA] = 1 mM, [NaN₃] = 1 mM, [GR] = 1 unit, and [catalyst] = 10-50 μM in sodium phosphate buffer (50 mM, pH 7) at 37°C. e) [GSH] = 0.5-3 mM, [H₂O₂] = 0.2-2 mM, [EDTA] = 1 mM, [GR] = 1 unit, [NADPH] = 0.25 mM in potassium phosphate buffer (50 mM, pH 7). f) [GSH] = 0.5-8 mM, [NADPH] = 0.25 mM, [EDTA] = 1 mM, [NaCl] = 100 mM, [NaN₃] = 1 mM, [GR] = 1 unit/mL, [catalyst] = 60 μM in potassium phosphate buffer (50 mM, pH 7) at 37°C.

1.3.2.3 Reproducing the catalytic tetrad using model peptides

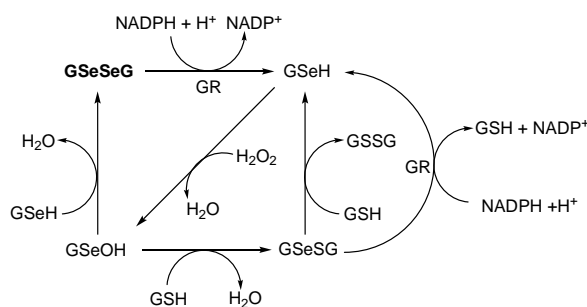
Later on, Iwaoka *et al.* have introduced the 4th amino acid of the catalytic tetrad to improve the catalytic activity of the peptidyl mimics. An additional asparagine was added at C terminus either directly bound the tryptophan (Table 3, entries 10 and 11) or with a glycine spacer (Table 3, entry 9) [83]. The incorporation of the glycine spacer appeared to be beneficial for the GPx-like activity. They have then head-to-tail cyclized these peptides using a N to S acyl migration followed by an intramolecular Sec-native chemical ligation (Sec-NCL) and an oxidation (Scheme 2) to afford the corresponding dimeric cyclized peptides (Table 3, entries 12-15). Again, the cyclic peptide possessing the Gln, Sec, Trp and Asn separated by a single residue (Table 3, entry 12) exhibited the highest initial rate among the series. More importantly, the activity was also higher than for the corresponding acyclic peptide suggesting that fixing the relative side chain positions is beneficial. In addition, this study reveals that Gln (Table 3, entry 15 vs. 12) plays a crucial role whereas the substitution of the Trp by an Ala (Table 3, entry 13 vs. 12) or of the Asn for an Ala (Table 3, entry 14 vs. 12) did not impact the kinetics in a large extent.



Scheme 2. Strategy for the synthesis of cyclic selenopeptides via tandem N-to-S acyl migration and intramolecular Sec-NCL. Reproduced by permission of The Royal Society of Chemistry [83].

1.3.2.4 Glutathione mimic

The diselenide dimer of selenogluthatione ($\text{H}_2\text{N}-\gamma\text{-Glu-Sec-Gly-OH}$)₂ (GSeSeG) has been also investigated for its GPx-like activity (Table 3, entry 16) [52]. Interestingly, it showed remarkable antioxidant properties even in absence of GSH. To explain such improved properties in comparison with primary sequence mimics that are of similar size (Table 3, entries 1-4), the authors have investigated in detail the possible reactions that occurred during the assay (scheme 3). They have shown that, among others explanations, the structural similarities of GSeSeG with GSSH enables its direct reduction by glutathione reductase GR leading to the reactive GSeH intermediate. This was not the case with ($\text{H}_2\text{N-Leu-Sec-Gly OH}$)₂ and ($\text{H}_2\text{N-Ser-Leu-Sec-Gly-Thr-OH}$)₂ which suggests that the carboxylate of the $\gamma\text{-Glu}$ residue is important for the regulation of the redox reactivity. Such effect has also been observed for small molecules reproducing GPx activity [87,88].



Scheme 3. Entire reaction mechanism for the antioxidative GPx activity of GSeSeG. Adapted from ref [52].

1.3.2.5 Peptides with a GSH binding site

The measured catalytic activity of the above-mentioned peptides was always quite low and the residues surrounding the Sec amino acid have only a weak influence. These model peptides were poorly efficacious most probably because they lack a GSH binding site. In the SecGPx structure published by Epp *et al.* [48], two arginines and a glutamine were presumably involved in the GSH binding through salt bridges and hydrogen bonds. This is why, Yin *et al.* have designed a peptide

involving the Sec residue, an asparagine, replacing the glutamine and two arginine residues [84]. They have shown that this peptide ((H₂N-Arg-Gly-Sec-Arg-Asn-OH)₂) exhibited quite good catalytic activity through a ping-pong mechanism similar to those of GPx (Table 3, entry 17). This peptide has been assayed in cells and in mice as an anticancer agent. It showed promising properties but the link between its anticancer properties and its GPx-like activity has not been yet clearly established [84].

Inspired by this peptide, a longer sequence with an Ala-Gln dipeptide added at the C-terminus has been studied [85]. This dipeptide has been added since it has been shown to decrease ischemia-reperfusion (I-R) injuries in rats most probably by preserving GSH content [89]. GPx-like kinetics of this peptide have not been investigated in detail but it has shown a higher GPx-like activity than the shorter peptide (Table 3, entry 18 vs. 17). It has been investigated in a rat model of liver I-R and has exhibited a protective effect after an intravenous injection. In hepatic I-R, ROS produced during the reperfusion are indeed responsible of oxidative damages and they are also involved in oxygen-radical-induced apoptosis [90]. Interestingly this peptide was efficacious on both pathways, which opens up new therapeutic opportunities [85].

1.3.2.6 Peptides selected from a phage display library

To confer enhanced GSH binding properties to the peptidyl mimics, Luo *et al.* have created a 15-mer phage display library and selected the phages with high affinity for GSH [76]. The sequences of the selected peptides were then determined by DNA sequencing of the phages. The C-terminal serine of the peptide (15P₁) possessing the best association constant with GSH was then chemically modified into a selenocysteine leading to the selenopeptide 15SeP₁ (Table 3, entry 19). This peptide exhibited a poor hydroperoxide affinity, consequently, the authors have mutated the first residues of the sequence with more hydrophobic residues that are often observed at the SecGPx catalytic site. The reported value of the second order catalytic rate of the resulting peptide 15SeP is extremely high, only 1 order of magnitude weaker than the native GPx1 (Table 3, entry 20). However, this extremely high GPx-like

activity has been questioned by Casi and Hilvert [86] that have published a much lower activity of the same peptide and no explanation was afforded to explain such discrepancies. Nevertheless, this peptide has been assayed for its antioxidant properties on rat epidermal cells submitted to a high H₂O₂ concentration. Luo *et al.* have shown that 15SeP significantly reduced lipids peroxidation, cells leakage and increased cells viability [76]. To note, this peptide is the only example of GPx mimic selected from a combinatorial library. Contrary to what has been done for the discovery of peptidyl SOD mimics, no combinatorial library on solid support associated with GPx activity-based screening has been reported so far to develop peptidyl GPx mimics [33].

1.3.3 *GPx mimics with superoxide dismutase-like activity*

To ensure a global protection from ROS in cells, antioxidant enzymes act cooperatively or synergistically: the balance between their antioxidant activities and their intracellular levels being very important. For example, a beneficial effect of a SOD-CAT conjugate has been described in a rat model of heart I-R injury whereas the SOD alone was found less efficacious or even detrimental at high doses [91]. Consequently, efforts have been devoted to the development of GPx mimics possessing also a SOD-like activity.

1.3.3.1 *Engineering proteins with a dual functionality*

A first example of a protein possessing such a dual activity has been reported by Luo *et al.* [92]. Serines of the pig blood Cu,ZnSOD have been chemically converted into selenocysteines by the already described 2-step procedure [61]. The resulting mutated SOD (Table 4) exhibited a low homogeneity as well as a low GPx activity. To circumvent this difficulty, Yu *et al.* have combined the fusion protein technology with the production of a seleno semi synthetic protein in bacteria supplemented in selenocysteine and deprived with cysteine [93]. They thus produced a Se-glutathione S-transferase (GST) fused with a SOD (Table 4). This construct showed both catalytic properties (SOD and GPx) and was able to reduce oxidation-induced damages in isolated

mitochondria. However, the applications of such big macromolecules that are expressed in inclusion bodies are very limited.

1.3.3.2 Miniaturization of dual functionality mimics

In an attempt to decrease the size and the complexity of the resulting objects, an approach that has been undertaken consists of conserving key sequences of the Cu,ZnSOD3 (residues 96-138 and 161-167) and replacing the sequence in between (residues 139-160) that has a more variable composition among SODs [94] with the peptide 15SeP from Luo *et al.* possessing a GPx activity [76]. The resulting sequence has then been slightly modified according to molecular modeling studies [95]. Since Val2 and Phe33 were found close to the Sec58, these amino acids have been replaced by Gln2 and Trp33 respectively to potentially reproduce the catalytic triad of the natural GPx. In addition, to produce this construct, the Cys12 has been mutated by an alanine to enable the incorporation of the selenocysteine at the desired position (Cys58) through the utilization of a bacterial cysteine auxotrophic strain cultured in absence of Cys in the growth medium and with a Sec supplementation. After peptide expression and cell lysis, CuSO₄ and ZnSO₄ were added into the cell-free extract to promote the metalation of the peptide. In one hand, the resulting Se-CuZn-65P peptide exhibited a SOD activity that was unambiguously associated with the presence of a Cu cation (Table 4). In the other hand, the GPx activity required the presence of the Sec residue and was improved when the two residues of the catalytic triad (Gln2 and Trp33) were introduced. Se-CuZn-65P has shown a protective effect of mitochondria against oxidative damage on a free radical damage system. In addition, this selenopeptide protects hepatocytes against ethanol-induced cytotoxicity [96]. It has been demonstrated that this effect occurred through the diminution of mitochondrial oxidative damages but also through the prevention of the oxidation-mediated apoptosis.

The design of this peptide was further modified to improve its internalization in cells. TAT (YGRKKRRQRRR), a cell penetrating peptide, has been added at the N-terminus of the Se-CuZn-65P leading

to Se76P [97]. This modification did not impact both catalytic activities (Table 4) but its effect on cell penetration was not clearly evidenced.

Table 4. GPx mimics with an additional SOD-like activity

	Activity		Kinetics		References
	SOD (U μmol^{-1})	GPx (U μmol^{-1})	SOD (μmol^{-1})	$k_{\text{cat}}/K_{\text{m,H}_2\text{O}_2}$ ($10^6 \text{ M}^{-1} \text{ min}^{-1}$)	
SOD ^[a]	$6.94 \cdot 10^5$	0			[92]
Mutated SOD ^[a]	$6.38 \cdot 10^5$	29			[92]
Se-GSTLu1-1-SOD ^[b]			12813	0.91	[93]
Se-CuZn-65P ^[c]	1254 U/mg	831		0.6	[95]
	1196 U/mg	112 U/mg			[96]
Se76P ^[a]	1278 U/mg	109 U/mg			[97]
SeCu32P ^[d]	$164 \pm 1.5 \cdot 10^{-3}$	146 ± 0.9		0.409	[98]
Se-SjGSTp35-Cu(II) ^[d]	1708 ± 21	2062 ± 22			[99]
GPx		5780		1000	[49,76]
SOD3	2970 U/mg				[100]

Reaction conditions: a) not reported. b) [GSH] = 1 mM, [H₂O₂] = 0.5 mM, [NADPH] = 0.25 mM, [EDTA] = 1 mM, [GR] = 1 U and [NaN₃] = 1 mM in phosphate buffer (50 mM, pH 7) at 37°C. Reaction performed with the Wilson method.[51] c) [GSH] = 1 mM, [H₂O₂] = 0.5 mM, [NADPH] = 0.25 mM, [GR] = 1 unit, and $m_{\text{catalyst}} = 0.5\text{-}2.5 \mu\text{g}$ in potassium phosphate buffer (50 mM, pH 7) at 37°C. Reaction performed with the Wilson method.[51] d) [GSH] = 1 mM, [H₂O₂] = 0.5 mM, [NADPH] = 0.25 mM, [EDTA] = 1 mM, [NaN₃] = 1 mM, [GR] = 1 unit, and [catalyst] = 10-50 μM in PBS buffer (50 mM, pH 7) at 37°C. Reaction performed with the Wilson method [51].

To improve the pharmacological potential of such mimics, Zou et al. [98] attempted to reduce the size of the peptide by fusing the 15P peptide described by Sun et al. [76] with a peptidyl copper complex developed in Luo's group [101]. The 32-mer peptide was synthesized by solid phase peptide synthesis with a Ser residue instead of the Sec [98]. The Ser was converted after purification into a Sec using the Wu and Hilvert method [62] and the peptide was then metalated. The resulting SeCu32P selenopeptide (Table 4) exhibited poor catalytic activities. However, this difunctional peptide showed a better protective effect on Vero cells submitted to ROS damage than the two monofunctional peptides, highlighting the importance of the synergism between SOD and GPx activities. An analogue of this dual activity peptide fused to a GST from *Schistosoma japonicum* GST (SjGST) has been expressed in *Escherichia coli* to generate a mimic with the three activities: SOD, GPx and GST [99]. After metalation and Sec introduction by the chemical modification

of serines, the resulting Se-SjGSTp35-Cu(II) exhibited higher SOD and GPx activities than SeCu32P and an additional GST activity (2209 ± 23 U/ μ M of enzyme). This represents the first example of a trifunctional enzyme.

1.4 Conclusion

To catalytically reduce hydroperoxydes into their corresponding alcohols, three partners are involved: hydroperoxide, GPx and GSH. Efficacious catalysis by GPx thus requires that the GPx binds both ROOH and GSH with high affinity. That is why, the development of GPx mimics reproducing such features is thus not very straightforward. This chapter has reviewed the different strategies, ranging from protein engineering to small peptide design, that have been developed to mimic GPx, which already appeared as an appealing strategy to fight against oxidative stress in the early 90's. However, contrary to what have been done for SOD mimics, only a limited number of studies has been devoted to the utilization of GPx mimics in biological relevant conditions, most probably because they lack stability and efficacy in intricate media such as cells or tissues. This highlights the urgent need to develop new strategies enabling the discovery of efficacious and stable GPx mimics.

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Abbreviations

CAT: catalase

CEOOH: cholesterol ester hydroperoxide

ChOOH: cholesterol hydroperoxide

DTT: dithiotreitol

GPCR: G-protein coupled receptors

GPx: glutathione peroxidase

Grx: glutaredoxin;

GSH: glutathione

GSSG: glutathione disulfide

HIV: human immunodeficiency virus

LOOH: lipid (fatty acid) hydroperoxide

NAD⁺: Nicotinamide adenine dinucleotide (oxidized form)

NADH: Nicotinamide adenine dinucleotide (reduced form)

PDI: protein disulfide isomerase

PLOOH: phospholipid hydroperoxide of different classes

PMSF: phenylmethanesulfonyl fluoride

RNA: ribonucleic acid

ROOH: small synthetic hydroperoxides

SOD: superoxide dismutase

Trx: thioredoxin