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Antioxidative glutathione analogues (UPF peptides) – molecular design, structure-activity relationships and testing the protective properties

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LIST OF ORIGINAL PUBLICATIONS

I **Ehrlich K**, Viirlaid S, Mahlapuu R, Saar K, Kullisaar T, Zilmer M, Langel Ü, Soomets U. Design, synthesis and properties of novel powerful antioxidants, glutathione analogues. Free Radic Res. 2007 Jul;41(7): 779–87.

II Vaher M, Viirlaid S, **Ehrlich K**, Mahlapuu R, Järvet J, Soomets U, Kaljurand M. Charaterization of antioxidative activity of novel non-toxic neuropeptides using capillary electrophoresis. Electrophoresis. 2006 Jul; 27(13): 2582–9.

III Mahlapuu R, Vaher M, **Ehrlich K**, Kaljurand M, Soomets U. Comparison of the stability of glutathione and related synthetic tetrapeptides by HPLC and capillary electrophoresis. J Pept Sci. 2006 Dec;12(12): 796–9.

IV **Ehrlich K**, Ida K, Mahlapuu R, Kairane C, Oit I, Zilmer M, Soomets U. Characterization of UPF peptides, members of the glutathione analogues library, on the basis of their effects on oxidative stress related enzymes. Accepted by Free Radical Research on March 20, 2009. Uncorrected proof.

Author's contribution:

I. The author participated in the design, synthesis, purification and MALDI-TOF MS analysis of the UPF peptides, measured the hydroxyl radical scavenging ability to a large extent and performed the peptide dimerization assay. The author was the main writer of the manuscript.

II. The author conducted the MALDI-TOF MS analysis, including the measurements of UPF peptides oxidation, participated in the UPF peptide synthesis, purification, contributed to the experimental design and the writing of the manuscript.

III. The author participated in peptide synthesis, purification and in hydroxyl radical elimination assays, and performed the RP-HPLC and MALDI-TOF MS analysis to assess the stability of UPF peptides. The author also participated in the writing of the manuscript.

IV. The author contributed to the enzyme activity assays and, especially, individually carried out the NAD(P)H oxidase measurements and introduced it to this lab. The author was the main writer of the manuscript.

ABBREVIATIONS

AD	Alzheimer's disease
ADS	antioxidant defence system
ARDS	acute respiratory distress syndrome
CAT	catalase
CE	capillary electrophoresis
cGPx	cytosolic glutathione peroxidase
COPT	chronic obstructive pulmonary disease
Cu,ZnSOD	copper,zinc-superoxide dismutase
CVD	cardiovascular diseases
DIEA	N,N-diisopropylethylamine
DMF	N,N-dimethylformamide
DMSO	dimethylsulphoxide
DPPH$^{\bullet}$	α,α-diphenyl-picrylhydrazyl radical
EDT	1,2-ethanedithiol
ESeH	selenol
ESeOH	selenic acid
ESeSG	selenenyl sulphide
Fmoc	9-fluorenylmethoxycarbonyl
GCL	glutamate-cysteine ligase
γ-GCT	γ-glutamylcyclotransferase
GGT	γ-glutamyl transpeptidase
GIGPx	gastrointestinal glutathione peroxidase
GPx	glutathione peroxidase
GR	glutathione reductase
GS	glutathione synthetase
GS$^{\bullet}$	glutathionyl radical
GSH	glutathione
GSSG	glutathione disulphide
GST	glutathione S-transferase
HOBt	hydroxybenzotriazole
HOCl	hypochlorous acid
L-012	(8-amino-5-chloro-7-phenylpyrido[3,4-d]pyridazine-1,4-(2H,3H)dione sodium salt
LDH	lactate dehydrogenase
MALDI-TOF MS	matrix-assisted laser desorption ionization time-of-flight mass spectrometry
MAP	model amphiphatic peptide
MnSOD	manganese-superoxide dismutase
MPO	myeloperoxidase
MTX	methotrexate
NAC	N-acetylcysteine
NO	nitric oxide
$O_2^{\bullet -}$	superoxide radical

$OH^{\bullet}$	hydroxyl radical
$ONOO^{-}$	peroxynitrite
OxS	oxidative stress
PD	Parkinson's disease
PDBu	phorbol 12,13-dibutyrate
pGPx	plasma glutathione peroxidase
PHGPx	phospholipid hydroperoxide glutathione peroxidase
PMNs	polymorphonuclear neutrophils
$R^{\bullet}$	carbon centred radical
RBCs	red blood cells
RNS	reactive nitrogen species
$RO^{\bullet}$	lipid alkoxyl radical
ROH	lipid alcohol
$ROO^{\bullet}$	lipid peroxyl radical
ROOH	lipid peroxide
ROS	reactive oxygen species
RP-HPLC	reverse-phase high performance liquid chromatography
RS	reactive species
SDS	sodium dodecyl sulphate
SOD	superoxide dismutase
SPPS	solid phase peptide synthesis
t-Boc	*tert*-butyloxycarbonyl
TBTU	2-(1H-bensotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate
TFA	trifluoroacetic acid
THA	terephthalic acid
TIS	triisopropylsilane
TRX	tioredoxin

INTRODUCTION

The metabolism of the human body constantly produces reactive species (RS) necessary for a number of physiological functions such as phagocytosis, signal transduction, gene expression, biotransformation, etc. As excessive quantities of RS are able to damage all principal biomolecules (proteins, DNA, and lipids), the human body and cells have an integrated antioxidant defence system (ADS) for neutralizing the RS overproduction. The ADS consists of RS-eliminating enzymes (superoxide dismutases, glutathione peroxidases, catalase, etc.) and low molecular weight compounds (glutathione, vitamin C, vitamin E, uric acid, bilirubin, etc.) and blood albumin. Among the non-enzymatic cellular antioxidants glutathione (GSH), found in millimolar concentrations in all mammalian cells, has a principal position. GSH is a cysteine containing tripeptide (γ-L-Glu-L-Cys-Gly). It is converted into an oxidized disulphide form (GSSG) during the elimination of reactive species. In addition to a significant antioxidative role, GSH has an impressive spectrum of biofunctions (xenobiotic detoxification, amino acid transport, maintenance of proteins' bioactive status, etc). The glutathione redox ratio (GSH/GSSG) is important for the redox-regulation of transcription factors, cell growth, differentiation and apoptosis. Therefore, any depletion of the cellular GSH pool is a serious threat for cellular homeostasis. Attenuated antioxidant defence or/and the production of the RS exceeding the capacity of the ADS leads to abnormal redox status and oxidative stress (OxS). Profound high grade OxS has an impact on the pathogenesis of many diseases including neurodegenerative ones (Parkinson's disease, Alzheimer's disease), cardiovascular diseases (CVD), sepsis, diabetes, cancer formation, HIV, ischemia/reperfusion injuries, etc. There is a body of evidence that these diseases/pathological states are related to a decreased glutathione pool and the GSH/GSSG ratio. A shift in the cellular redox balance (based principally on the glutathione redox ratio) towards an oxidized state is often an initiator of pathological processes. Supporting/maintaining the normal functionality of the glutathione system is a reasonable goal under OxS-related conditions. The elevated production of RS can also be accompanied by aging, stress or strenuous physical exercise.

Different strategies to increase/maintain GSH cellular levels have been tested. The administration of GSH itself is not reasonable because peptidic GSH is rapidly degraded in the gut and circulation. In addition, glutathione is poorly taken up by most of the cells and does not cross the blood-brain barrier. One strategy is based on the use of amino acid cysteine precursors needed for GSH *de novo* synthesis, as the availability of cysteine is the limiting factor. As cysteine itself is toxic at higher concentrations (it becomes pro-oxidant), the precursor of cysteine, N-acetylcysteine (NAC), is most often used. This approach expects that the glutathione synthesis apparatus is unimpaired and has high functionality. A new strategy is the usage of glutathione analogues. Designed glutathione analogues for supporting the glutathione system should be stable, non-toxic and desirably with better antioxidativity compared to

glutathione. In this thesis the design and synthesis of a glutathione analogues library (arbitrarily called UPF peptides), their free radical scavenging properties, and their stability and interactions with principal antioxidative and prooxidative enzymes will be analyzed. It is noteworthy that one of the members of the library (UPF1) has already shown protective effects in animal experimental models.

REVIEW OF LITERATURE

1. Oxidative stress

1.1 Basic terms related with oxidative stress

Oxidative stress (OxS) is classically defined as an imbalance between antioxidants and oxidative stressors with a shift towards oxidative stressors that may lead to potentially harmful processes (Halliwell and Gutteridge 1999; Packer and Cadenas 2007; Sies 1991). In a broader context, an oxidative stressor can be any chemical or physical factor that causes OxS in the human body. Exogenous initiators of OxS can be smoking, exhaust gases, excess of Fe, Cu and other heavy metals in the food, and radiation and xenobiotics (including drugs) that diminish the antioxidant supplies in the organism. Principal oxidative stressors are reactive oxygen species (ROS) and reactive nitrogen species (RNS) as the other factors (including ions of transition metal) mediate their effect via the promotion of the formation of ROS and RNS. Damages caused by RNS are often also referred to as nitrosative stress. The shift in the thiol system (largely based on GSH/GSSG) towards a more oxidized status is one of the principal features of OxS. Thus it has been recently suggested to refine the definition of OxS as "disruption of redox signaling and control" (Jones 2008; Sies 2007). Generally, an antioxidant is defined as a substance (including enzymes) that can prevent, interrupt or repair oxidative damage. A comprehensive definition of an antioxidant could be formed as: "any substance that, when presented at low concentration compared with those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate" (Halliwell and Gutteridge 1999). RS involves free radicals and non-radical reactive species. Free radicals are the species that contain at least one unpaired electron and are capable of independent existence. Free radicals can be nitrogen-, sulphur-, oxygen- or carbon-centred. Well-known free radicals in the human body that have both physiological impact and are related to pathological events are superoxide radical ($O_2^{\bullet -}$), hydroxyl radical ($OH^{\bullet}$), lipid alkoxyl and peroxyl radicals ($RO^{\bullet}$ and $ROO^{\bullet}$, respectively) and nitric oxide (NO) as RNS. In general, free radicals are characterized by an extremely short existence and high reactivity. ROS also include non-radical species as H_2O_2 and hypochlorous acid (HOCl), and the well-known non-radical RNS is peroxynitrite ($ONOO^-$). ROS and RNS are continuously produced by the metabolism of the human body. They are necessary for a number of biofunctions, as in the host defence by phagocytic cells, biosynthesis reactions, in signaling processes, etc. Under some conditions RS are produced in excess and ADS is incapable of controlling their production and effects. RS as well as the oxidative shift in the glutathione redox state (GSH/GSSG) in certain cell types have been implicated in the activation of transcriptional factors, such as AP-1 and NF-κB. (Droge 2002a; Hehner *et al.* 2000). This is important, as NF-κB regulates the expression of key cytokines

and chemokines that further modulate the inflammatory response. Thus the excessive production of RS does not only damage biomolecules nearby, but it also leads to the activation of several major signaling pathways (Finkel and Holbrook 2000).

1.2 Endogenic sources of reactive species

Superoxide radical is a one-electron reduction product of oxygen. By further reactions superoxide radicals are converted to RS, which have higher reactivity. One of the considerable producers of $O_2^{\bullet -}$ are phagocytic and vascular NAD(P)H oxidases (cf in a special chapter). Superoxide radicals are also produced in the respiratory chain and this is favoured when mitochondrial damage occurs (McCord 2000). Oxidative phosphorylation and ATP synthesis in the mitochondria uses about 90% of the oxygen consumed by the human body. At physiological levels, it has been proposed that about 1–3% of the oxygen reduced in the mitochondria may be transformed into $O_2^{\bullet -}$ (Halliwell and Gutteridge 1999). This creates a good basis for the generation of ROS, which in turn needs effective ADS. Other superoxide radical producers are auto-oxidation reactions of catecholamines and xanthine oxidase, an enzyme needed for purine catabolism.

Hydroxyl radical is the most highly reactive free radical in the human body. It can react with proteins, lipids and DNA, disturbing their biofunctions and initiating free radical chain reactions. Because of the extremely high reactivity and short life-span, hydroxyl radical only reacts with the molecules in its immediate vicinity. As far as is known there is no enzymatic mechanism for hydroxyl radical elimination. Hydroxyl radical can be formed via a Fenton reaction or after exposure to ionizing γ-irradiation. The Fenton reaction (reaction 1) is the major mechanism of hydroxyl radical generation from hydrogen peroxide catalyzed by transition metal ions (concerning that, human body iron as well as copper oxidation states have an impact). Free iron ion is an especially potent oxidative stressor causing the production of hydroxyl radicals (Gate *et al.* 1999; Halliwell and Gutteridge 1999).

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^{-} \quad (1)$$

Although Fe^{2+} and Cu^{+} have higher pro-oxidative potential, Fe^{3+} and Cu^{2+} can take part in radical generation as well. In the presence of reducing factors (ascorbic acid, NAD(P)H, cysteine), the respective conversions of Fe^{3+} and Cu^{2+} to Fe^{2+} and Cu^{+} take place. In a Haber-Weiss reaction, hydrogen peroxide and superoxide radical are involved in hydroxyl radical generation, whereas both iron and copper ions are the potential mediators of radical generation (reactions 2–6).

A Haber-Weiss reaction in the case of ferrum:

$$Fe^{3+} + O_2^{\bullet -} \rightarrow Fe^{2+} + O_2 \quad (2)$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^{-} \quad (3)$$

A Haber-Weiss reaction in the case of copper:

$$Cu^{2+} + O_2^{\bullet-} \rightarrow Cu^{+} + O_2 \quad (4)$$
$$Cu^{+} + H_2O_2 \rightarrow Cu^{2+} + OH^{\bullet} + OH^{-} \quad (5)$$

Taking reactions 2–5 together:

$$H_2O_2 + O_2^{\bullet-} \xrightarrow{\text{Fe/Cu catalyst}} OH^{\bullet} + OH^{-} + O_2 \quad (6)$$

In addition, Fe^{3+} can intermediate its toxicity by reacting with hydrogen peroxide (reaction 7), resulting in the formation of superoxide radical and Fe^{2+}, which can subsequently participate in the Fenton reaction described above. It must be considered that this reaction is much slower than the reaction of H_2O_2 with Fe^{2+} (Halliwell and Gutteridge 1999).

$$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + O_2^{\bullet-} + 2H^{-} \quad (7)$$

Hydrogen peroxide is mainly formed via an elimination of superoxide radical by superoxide dismutases (SOD). Although less reactive, hydrogen peroxide is highly diffusible and can cross plasma membranes and cause damaging impact farther from the place it originally generated. GSH, together with glutathione peroxide (GPx), is needed for the elimination of an excess of hydrogen peroxide.

Hypochlorous acid is a stable powerful oxidizing agent, which can oxidize several targets such as thiols, ascorbate, NAD(P)H and chlorinate DNA bases and tyrosine residues in proteins. Physiologically, HOCl is produced from hydrogen peroxide and chlorine ions by myeloperoxidase (MPO) during the respiratory burst in the activated neutrophils (reaction 8).

$$H_2O_2 + Cl^{-} \xrightarrow{\text{MPO}} HOCl + OH^{-} \quad (8)$$

NO and $ONOO^{-}$ are the most common nitrogen species, whereas NO is a free radical. NO is produced from L-arginine by nitric oxide synthases and works as a signal molecule for the CNS, as a relaxation factor for smooth muscles in blood vessels, etc. Superoxide can rapidly react with NO and form a toxic $ONOO^{-}$. This reaction gains clinical importance when the overproduction of superoxide radical occurs. The depletion of NO by superoxide radicals leads to impaired vascular relaxation and hypertension.

The next class of free radicals, carbon-centred ($R^{\bullet}$), are generated by an attack by hydroxyl radicals on fatty acids, which in a further reaction with oxygen gives peroxyl radicals. Alkoxyl and peroxyl radicals ($RO^{\bullet}$ and $ROO^{\bullet}$,

respectively) are major intermediates in lipid peroxidation and can be formed as a result of the decomposition of lipid peroxides (ROOH).

Thiols (especially GSH) act mainly as antioxidants; however, the possibilities for their transformation into sulphur centred radicals still exist. The initiators can be carbon-centred radicals, oxygen radicals ($OH^{\bullet}$, $RO^{\bullet}$, $ROO^{\bullet}$, $O_2^{\bullet -}$), nitrogen dioxide or peroxynitrite, transition metal ions or the homolytic fission of disulphides. For example, GSH reacts with $OH^{\bullet}$ as follows, resulting in glutathionyl radical formation ($GS^{\bullet}$) (Halliwell and Gutteridge 1999):

$$GSH + OH^{\bullet} \rightarrow GS^{\bullet} + H_2O \quad (9)$$

There are many ways as to how free radicals and other RS can react with each other and attack the biomolecules. However, this work refers only to the most central RS reactions in the human body and the most important ones, considering the current research.

1.3 NAD(P)H oxidase

NAD(P)H oxidase is a major producer of superoxide by the phagocytic cells. Thus, current study is also focused on the possibility of controlling superoxide overproduction using UPF peptides as NAD(P)H oxidase inhibitors. This membrane-associated oligomeric enzyme catalyses the reduction of molecular oxygen using NADPH as the electron donor. When the enzyme is dormant in resting cells, two subunits (gp91phox, p22phox) are located in the membrane and three in the cytosol (p47phox, p67phox, p40phox) in a complex with a small GTPase Rac (Groemping and Rittinger 2005). During the activation process, cytosolic subunits migrate to the membrane and assemble the active enzyme complex, whereas serine phosphorylation in p47phox seems to be the initial step (Roos *et al.* 2003). However, phagocytic NAD(P)H oxidase is a critical component of a host defence system; its over-reactivity can cause an excessive production of free radicals. Emerging evidences suggest that patients with sepsis suffer from profound OxS. According to this, antioxidants have been considered as an adjuvant component of sepsis treatment (Cadenas and Cadenas 2002; Victor *et al.* 2005).

An alternative form of NAD(P)H oxidase is located in vascular smooth muscle cells and in endothelial cells. This vascular NAD(P)H oxidase has a similar structure to phagocytic NAD(P)H oxidase, but gp91phox is replaced with respective homologues called Nox proteins. Differently from phagocytic NAD(P)H oxidase, which releases superoxide radicals in bursts, vascular NAD(P)H oxidase produces continually low levels of superoxide radical, rather intracellularly (in vascular smooth muscle cells), and has a regulatory role (Griendling *et al.* 2000; Souza *et al.* 2001). Increased superoxide radical production by vascular NAD(P)H oxidase is related to several CVD, such as atherosclerosis, hypertension and heart failure (Cai *et al.* 2003; Hamilton *et al.*

2002; Murdoch *et al.* 2006). Vascular NAD(P)H oxidase is suggested as the main source of superoxide radical in vascular cells and myocytes (Griendling *et al.* 2000). NAD(P)H oxidase inhibitors are potential leads for the design of novel anti-inflammatory substances (Van den Worm *et al.* 2001). Molecules such as peptide PR-39, apocynin and its analogues have been tested to decrease NAD(P)H oxidase over-reactivity in pathological conditions (Ikeda *et al.* 2001; Van den Worm *et al.* 2001).

2. Antioxidant defence system of the human body

2.1 Classification of antioxidants

To control the level of RS, the human body has an integrated network of antioxidant defence (ADS), consisting of enzymes, low molecular weight substances and blood albumin. Most often antioxidants impede the formation of new RS by converting the existing ones into a less toxic form or by preventing the conversion of molecules into free radicals. For the best overview antioxidants can be divided according to their cellular location and water solubility. Glutathione is the major intracellular low molecular weight antioxidant. Another important intracellular water soluble antioxidant is ascorbic acid. Intracellularly acting free radical eliminating enzymes constitute the first line of defence against RS.

Superoxide dismutase (SOD) – converts superoxide radical into hydrogen peroxide

$$O_2^{\bullet -} + O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2 + O_2$$

Catalase (CAT) – catalyses decomposition of hydrogen peroxide into water and oxygen

$$H_2O_2 + H_2O_2 \rightarrow 2H_2O + O_2$$

Glutathione peroxidase (GPx) – removes hydrogen peroxide and fatty acid hydroperoxides (ROOH) in cooperation with glutathione

$$H_2O_2 + 2GSH \rightarrow GSSG + 2H_2O$$
$$ROOH + 2GSH \rightarrow GSSG + H_2O + ROH$$

It is accepted that extracellular forms of SOD and GPx exist but have a limited relevance compared to respective intracellular enzymes. Vitamin E, ubiquinol and carotenoids are the main antioxidants in a lipid environment (cell membranes, lipoproteins), whereas vitamin E carries a leading role. Blood plasma contains several water soluble antioxidants, such as ascorbic acid, uric acid, albumin, bilirubin, apotransferrin and ceruloplasmin. As the activities of GPx and MnSOD are measured in this study, special emphasis on these enzymes will be further provided.

2.2 Manganese-superoxide dismutase

Three forms of superoxide dismutases exist in mammalian cells: a copper,zinc-superoxide dismutase (Cu,ZnSOD), a manganese-superoxide dismutase (MnSOD) and an extracellular superoxide dismutase. Most of the Cu,ZnSOD is located in cytosol, but some quantity appears to be in lysosomes, the nucleus and the space between the inner and outer mitochondrial membranes (Halliwell and Gutteridge 1999). Superoxide dismutases are the primary enzymes that keep cellular free radical production under control (Perry *et al.* 2007). All superoxide dismutases catalyse the same dismutation reaction of superoxide. MnSOD is the principal enzymatic antioxidative defence in mitochondria. A severe reduction of succinate dehydrogenase and aconitase (both are the tri-carboxylic acid cycle enzymes) activities were detected in MnSOD deficient mice, which indicates that MnSOD is crucial for maintaining the activity of mitochondrial enzymes, related to energy production (Li *et al.* 1995). Human liver MnSOD is composed of four identical subunits, in which one cysteine (Cys196) is readily reactive towards thiols whereas the other (Cys140) is hidden inside the molecule (Matsuda *et al.* 1990). Many links between the depletion of the GSH pool and neurodegenerative diseases have been found, but information about glutathione level as one of the regulators of MnSOD activity in brain mitochondria is quite limited (Bharath *et al.* 2002; Chinta *et al.* 2006).

2.3 Glutathione peroxidase

Only GPx is able to eliminate an excessive amount of both H_2O_2 and lipid hydroperoxides in the cells (Dickinson and Forman 2002). During this process, GSH is converted to GSSG. GPx-s are the family of selenoproteins, whereas the four GPx isoenzymes described below, found in mammals, have a central role in peroxide elimination (Jefferies *et al.* 2003). The isoenzymes differ from each other by substrate specificity and by location. Cytosolic GPx (cGPx, GPx1; EC 1.11.1.9), as a classical GPx, is abundantly found in various tissues and blood cells and is able to reduce H_2O_2 and organic hydroperoxides, but is unable to detoxify phospholipid hydroperoxides. Gastrointestinal GPx (GIGPx, GPx2) is similar to cGPx by its substrate specificity. Phospholipid hydroperoxides are eliminated by phospholipid hydroperoxide glutathione peroxidase (PHGPx, GPx4), which is believed to be cytosolic and partly membrane bound, or by extracellular plasma GPx (pGPx, GPx3). Both of these enzymes are also able to eliminate H_2O_2 (Ren *et al.* 2001). PHGPx is ubiquitously expressed in the brain and testis (Maher 2005). cGPx, GIGPx and pGPx consist of four subunits, whereas PHGPx is a monomer. Each subunit contains one atom of selenium at its active site. The small size and hydrophobic nature of PHGPx are advantageous for interaction with lipid membranes (Brigelius-Flohe 1999). The selenol of a reduced selenocysteine molecule (ESeH) is oxidized by the hydroperoxides to form a selenic acid (ESeOH), which further reacts with GSH, resulting in

water and selenenyl sulphide (ESeSG). A second molecule of GSH attacks the sulphur of ESeSG, GSH disulphide (GSSG) is produced and the initial selenol (ESeH) is regenerated (Figure 1). GPx and catalase (CAT) are both vital for removing H_2O_2, but only GPx is able to eliminate lipid hydroperoxides. Most animal tissues have both CAT and GPx activity, but skeletal muscle, spermatozoa and certain brain regions contain low levels of CAT and more GPx. The liver has an extra high capacity of glutathione system for H_2O_2 removal. In red blood cells (RBCs), normal and low production of H_2O_2 are controlled by GPx; however, when H_2O_2 levels have risen, CAT becomes more important. GPx and CAT also possess different subcellular locations – so the removal of H_2O_2 also depends on where it is produced. CAT is largely located in the peroxisomes, whereas GPx is distributed in the cytosol and to some extent in the matrix of mitochondria. Mitochondria lack CAT except in the heart (Halliwell and Gutteridge 1999). Finally, one can conclude that H_2O_2 formed by cytosolic CuZnSOD and by mitochondrial MnSOD are first of all neutralized by GPx.

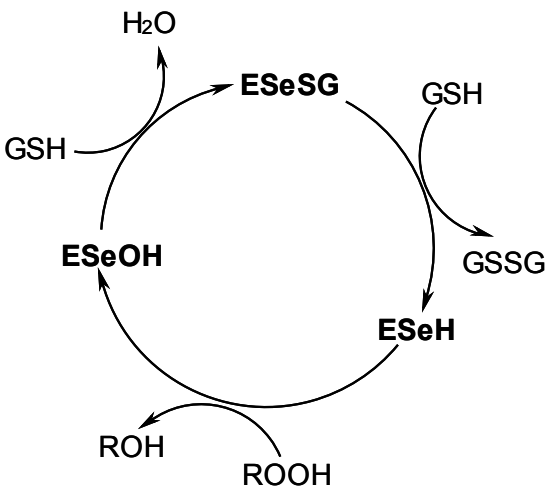

Figure 1. Reaction mechanism of peroxide elimination by GPx.

3. Glutathione

3.1 Characteristics and the metabolism of glutathione

Glutathione (GSH) carries an important role in the human body antioxidant defence system, as it is the most prominent low molecular weight thiol that occurs in millimolar range in most cells of the human body. Glutathione is a tripeptide composed of amino acids glutamate, cysteine and glycine (γ-L-Glu-L-Cys-Gly), and it has two characteristic structural features: a γ-glutamyl linkage

and a sulphydryl group. The liver is the main organ of glutathione synthesis, whereas the kidneys represent the high breakdown activity of glutathione and its conjugates. Glutathione level is also higher in the spleen, lens, erythrocytes and leukocytes. The concentration of glutathione in the cytosol is 0.5–10 mM (Pastore *et al.* 2003), whereas plasma concentration remains at a micromolar level (5–50 μM) (Griffith 1999). The GSH/GSSG ratio is normally considered to be 100:1. Some compartments of the cell, such as endoplasmatic reticulum, require a higher oxidizing status (from 3:1 to 1:1) to fulfil their biofunctions. Such a small GSH/GSSG ratio is thought to be necessary for the correct disulphide bond formation of proteins.

The GSH/GSSG ratio (also called the glutathione redox buffer for cells) can easily decrease if supplies of intracellular glutathione are constantly used for RS elimination. Reduced glutathione is also utilized for the conjugation of several substances, and these conjugates are excreted out of cells, which also contributes to the loss of intracellular GSH supplies. In addition, cells are trying to keep a normal GSH/GSSG ratio via GSSG efflux to extracellular compartments, although this leads again to a decrease in the total glutathione pool. Cells possess several mechanisms for maintaining the normal GSH/GSSG ratio and GSH levels. First, GSH is regenerated back from GSSG by the action of glutathione reductase (GR), which uses NADPH as a reducing power (Figure 2). Glutathione is *de novo* synthesized from glutamic acid, glycine and cysteine, whereas the availability/level of cysteine is the rate-limiting. The synthesis of glutathione takes place in cytoplasm and is catalyzed sequentially by two ATP-dependent cytosolic enzymes (Figure 3). The first and rate-limiting enzyme, glutamate-cysteine ligase (GCL), converts glutamic acid and cysteine into dipeptide γ-glutamylcysteine. The second enzyme, glutathione synthetase (GS), completes glutathione synthesis and is responsible for the addition of glycine to γ-glutamylcysteine dipeptide (Dickinson and Forman 2002). GCL consists of two subunits – a heavy catalytic subunit and a regulatory light subunit – and its activity is suppressed by the feedback inhibition of glutathione. Physiologically, the synthesis of GSH is upregulated by oxidative stimuli as an adaptive defence response. In addition to glutathione analogues and precursors, there are substances that can elevate intracellular glutathione level by upregulating its synthesis. Some antioxidants also mediate their influence through GCL. For example, in human vascular endothelial cells melatonin has been shown to upregulate the expression of GCL, the rate limiting enzyme of GSH synthesis (Urata *et al.* 1999). Apocynin, a NAD(P)H oxidase inhibitor, has also been shown to increase the GCL activity and mRNA expression in human alveolar epithelial cell culture (A549) (Lapperre *et al.* 1999).

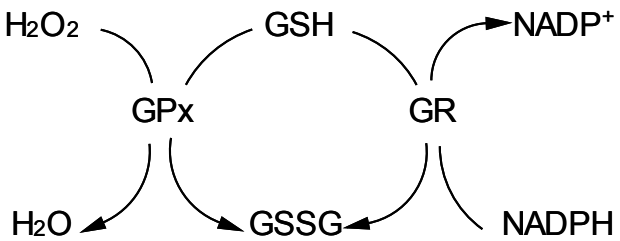

Figure 2. Peroxde elimination and GSH regeneration.

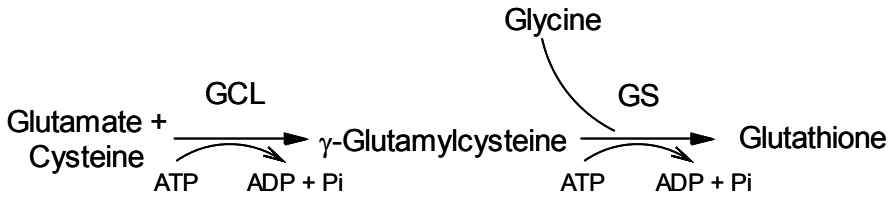

Figure 3. *De novo* synthesis of glutathione.

The breakdown of GSH, GSSG and GSH-conjugates is mediated by the enzyme called γ-glutamyl transpeptidase (GGT), located in the cell membrane. GGT removes the γ-glutamyl moiety from the glutathione and produces dipeptide Gly-Cys or Gly-Cys-conjugate, respectively. Removed glutamate is transported to an acceptor, which is an amino acid or other dipeptide. Extracellular dipeptidases hydrolyse the bond between Cys and Gly, resulting in free amino acids. In the case of Gly-Cys-conjugates, the conjugated part remains bound with cysteine. Formed compounds are further taken up by specific transporters and used for glutathione resynthesis (Franco *et al.* 2007) (Figure 4).

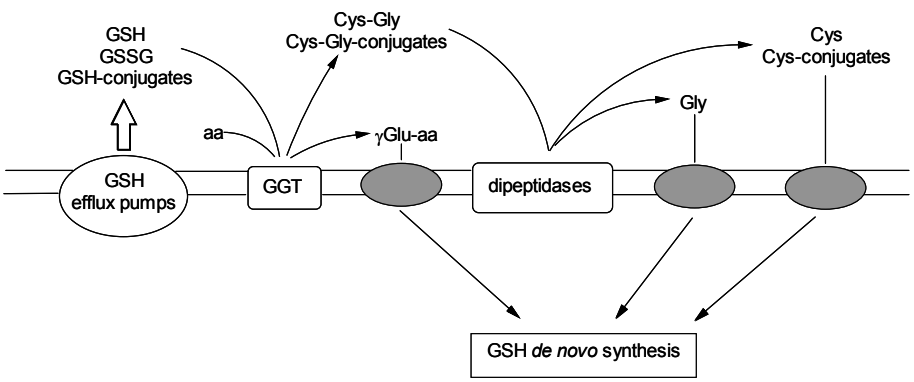

Figure 4. Glutathione efflux and the reusing of its components for *de novo* synthesis. (Modified from Franco *et al*. 2007).

Cellular organelles have distinct glutathione pools. The GSH pool in the nucleus is considered to be independent from cytoplasm, because it is not completely depleted by buthionine sulfoximine, a specific inhibitor of GCL and glutathione synthesis (Schafer and Buettner 2001). Approximately 10–15% of total cellular glutathione is localized in mitochondria, represented in similar concentration, than in the cytosol. As mitochondria lack the enzymes for glutathione synthesis, the mitochondrial glutathione level is maintained by its transport from cytosol (Pastore *et al.* 2003). The mitochondrial inner membrane is the main barrier for glutathione transport (Sheu *et al.* 2006). In the kidneys, GSH is transported into mitochondria via carrier-mediated way or by diffusion. Eight anion carriers, which could possibly transport glutathione, have been found in the mitochondrial inner membrane (Lash 2006). Additionally, some evidence suggests that there is no transport mechanism for GSSG efflux, which makes mitochondria especially sensitive towards the accumulation of GSSG in the case of OxS (Liang and Patel 2006).

3.2 Biofunctions of glutathione

In addition to being the principal cellular low molecular weight antioxidant, glutathione has broad spectra of biofunctions (Figure 5). Antioxidant function is executed via non-enzymatical reactions with certain RS (hydroxyl radical, hypochlorous acid, peroxynitrite) or through the elimination of peroxides being a co-factor in GPx (Cnubben *et al.* 2001; Halliwell and Gutteridge 1999). During this process, GSH oxidizes to dimeric form (GSSG). Cellular GSH depletion changes the glutathione redox status in the cell, which is defined as the GSH/GSSG ratio. Two other important redox systems inside the cells are $NADPH/NADP^+$ and tioredoxin (TRX_{red}/TRX_{ox}). The abundance of GSH makes it the major determinant of intracellular redox homeostasis. The concentration of glutathione is about 500–1000-fold higher than TRX and NADPH (Filomeni *et al.* 2002). The redox ratio of glutathione is involved in the modulation of redox-sensitive proteins activity (including some transcription factors as AP-1 and NF-κB) and regulates cell growth, differentiation, apoptosis and response to stress factors (Filomeni *et al.* 2002; Jefferies *et al.* 2003; Schafer and Buettner 2001). Intracellular glutathione level is determined by the synthesis, consumption and regeneration of GSH and the efflux of its conjugates and GSSG.

GSH as a nucleophile is able to react directly with endogenous and exogenous electrophilic compounds. The majority of these detoxification reactions are mediated by glutathione S-transferases (GST), an enzyme which conjugates the thiol group of glutathione to the xenobiotics (Dickinson and Forman 2002). Arising adducts are actively secreted out of the cell and degraded analogically to GSH by GGT, resulting in cellular glutathione pool depletion. After removal of γ-glutamyl moiety and the breakdown of the peptide bond between Cys-Gly, the conjugated part remains attached to Cys. The amino group of cysteinyl

residue will be acetylated to mercapturic acid, which is an excretion form of glutathione conjugates in the kidneys. GSH helps also to maintain the sulphydryl groups of coenzyme A and many proteins, including hemoglobin, in the functional reduced form. Proteins have free sulphydryl groups, which may react with low molecular weight thiols and form mixed disulphides. This process with the accompanied formation of disulphide bridges is called S-thiolation. As glutathione is the most abundant low molecular weight thiol in the cells, then most of the S-thiolation reactions are thiol-disulphide exchanges between protein cysteines and oxidized glutathione. This phenomenon, known as S-glutathionylation, also takes place in basal conditions, but its rate is increased when the GSSG level has risen due to OxS. (Ghezzi 2005).

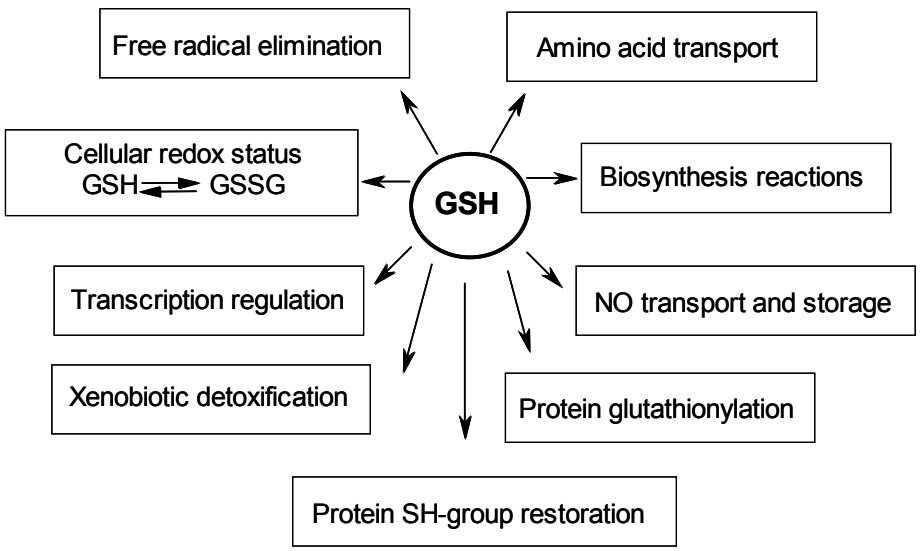

Figure 5. Biofunctions of glutathione.

The role of S-glutathionylation is the protection of proteins against irreversible oxidation and the regulation of their function. NO can form nitrosothiols with cysteine residues of protein and with low molecular weight thiols as glutathione, which plays an important role in the transport, storage and metabolism of NO (Hogg *et al.* 1996). Ascorbate is transformed to dehydro-ascorbate after the regeneration of α-tocopherol from α-tocopheryl radicals, which are formed from vitamin E during free radical elimination. Glutathione is a co-factor for the enzyme dehydroascorbate reductase, which converts dehydroascorbate back to ascorbate (Pastore *et al.* 2003). The latter is one example of the integration of an antioxidative system. Glutathione also suppresses the formation of methemoglobin. Glutathione is required for the synthesis of proteins, nucleic acids, leukotrienes and prostaglandins. The γ-glutamyl moiety

of glutathione is proposed to participate in the amino acid transport mediated by the γ-glutamyl cycle. After the breakdown of glutathione or its conjugates by GGT, γ-glutamyl derivatives are formed, consisting of the glutamyl moiety and its acceptor amino acid. These derivatives can be substrates for membrane-bound γ-glutamylcyclotransferase (γ-GCT), resulting in 5-oxoproline and corresponding amino acid, which is transported through the membrane with the help of glutamate. Finally, 5-oxoproline is converted to glutamate by the 5-oxoprolinase.

3.3 Glutathione depletion and pathological states

Both GSH depletion and high-grade OxS stress occur under a variety of conditions. RS contribute to the development of a wide spectrum of pathological states: several chronic diseases (CVD, neurodegenerative diseases, diabetes, tumors, HIV) and acute clinical conditions (inflammation, infarction, stroke, organ transplantation, ischemia/reperfusion injury, renal failure, lung injuries, complications of surgical operations) (Bharath *et al.* 2002; Emerit *et al.* 2004; Franco *et al.* 2007; Gate *et al.* 1999; Jefferies *et al.* 2003; Townsend *et al.* 2003; Valencia *et al.* 2002). The elevated production of RS is also shown in the case of stress, aging and exhaustive physical exercise (Droge 2002b; Finaud *et al.* 2006; Finkel and Holbrook 2000). Some of the pathological states are especially directly related to glutathione depletion, although the risk of decreasing glutathione supplies is always accompanied with high grade OxS.

Compared with other organs in the body the brain is especially susceptible to OxS, because of its high oxygen consumption, abundant iron and unsaturated fatty acid content, which are good targets for lipid peroxidation. The human brain comprises only 2% of the body weight but utilizes 20% of the oxygen consumed by the body (Pastore *et al.* 2003). Elevated markers of lipid peroxidation have been found in the specific brain regions of Parkinson's disease (PD) and Alzheimer's disease (AD), clearly indicating the damage by OxS. Although definite signs of damage caused by ROS are present in specific brain regions, the question remains – can these signs be a cause or consequence of neurodegeneration (Andersen 2004)? From all of these neurodegenerative diseases, PD seems to have the most direct causative link with glutathione depletion. The pathogenesis of PD is multifactorial, but two important hypotheses consider the shifted redox state towards OxS and reduced energy production accompanied by deficient mitochondrial activity (Bains and Shaw 1997). GSH depletion in the substantia nigra and in the dopaminic neurons of this area is considered to be one of the earliest indicators of neurodegeneration, and the extent of depletion is correlated with the severity of the disease (Andersen 2004; Pearce *et al.* 1997). Some evidence indicates that glutathione depletion may even precede the loss of reduced energy production, accompanied by deficient mitochondrial activity (Andersen 2004; Dexter *et al.* 1994).

AD is the most common cause of dementia in elderly people, characterized by progressive neuronal loss. The predominant hypothesis of AD development is based on amyloid-β-peptide neurotoxicity, at least partially mediated by free radical generation (Pappolla *et al.* 1998). Elevated levels of GSSG are detected in the brain tissue of AD patients (Pocernich *et al.* 2000), especially in the region of the frontal inferior cortex with an accompanied decrease in the GSH/GSSG ratio (Karelson *et al.* 2001).

A very common situation where the damaging effect of ROS and RNS occurs is the ischemia/reperfusion. Ishemia/reperfusion is serious complication in organ transplantation, myocardial infarction and stroke (Droge 2002a). Most of the ROS-mediated injuries are caused when the blood flow of ischemic tissues is re-established. It has been shown that glutathione has a critical role in the protection of myocardial cells in short term reversible ischemia/reperfusion (Blaustein *et al.* 1989; Ozer *et al.* 2005). Thus, the antioxidants are proposed to ameliorate myocardial ischemia/reperfusion injury. Figure 6 describes a mechanism for how OxS is contributed as a very early event to the pathophysiological continuum (Dzau *et al.* 2006).

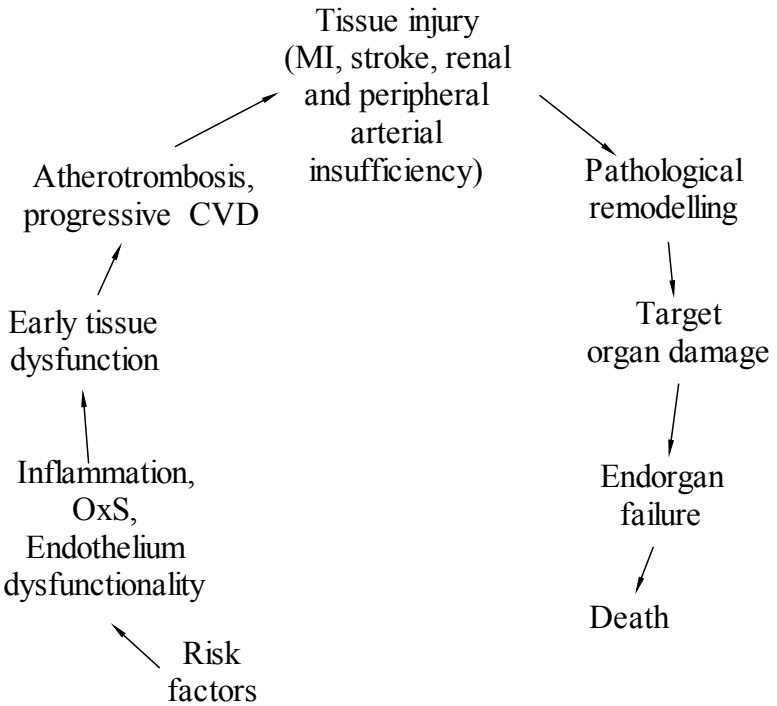

Figure 6. Cardiovascular and renal pathophysiological continuum (Dzau *et al.* 2006).

Critical diseases such as sepsis and acute respiratory distress syndrome (ARDS) are also characterized by high grade OxS and severe redox imbalance, which may finally exhaust the reduced glutathione supplies (Biolo *et al.* 2007; Brealey

et al. 2002). In clinical trails, the GSH precursor NAC has most often been used for replenishing the depleted glutathione stores. Although evidence indicates the close relationship of glutathione depletion and sepsis development, clinical trials of NAC supplementation in the case of septic shock and ARDS patients have given mixed results (Crimi *et al.* 2006).

As a central antioxidant, GSH protects cells from carcinogens by preventing RS-induced DNA damage. Additionally, in cooperation with GST, glutathione participates in xenobiotics detoxification, which may be potential cancerogens. GST polymorphisms resulting in decreased enzyme activity are lately linked to the progression of several cancer types (Franco *et al.* 2007; McIlwain *et al.* 2006). Hepatic glutathione supplies can be depleted as the result of alcoholism, hepatitis C or xenobiotics, most commonly acetaminophen.

Diabetes mellitus is a metabolic disease characterized by a diminished production of insulin (type 1) or by a resistance to its effects (type 2), resulting in non-physiologically high blood glucose levels. A poorly controlled elevation of blood glucose levels will finally lead to typical complications of diabetes – vascular and peripheral nerve damage and retinal degeneration. A large body of evidence shows that the enhanced production of RS promotes the development of diabetes and its complications, but still the following is unknown: is the OxS the primary event or only a secondary phenomenon of the tissue damage? Several complications of diabetes such as peripheral neuropathy, angiopathies- and atherosclerosis-mediated tissue hypoxia and gastric damage, have been shown to be associated with GSH depletion (Franco *et al.* 2007). The eye expresses a naturally high activity of protection by glutathione, but in the case of diabetic retinopathy it is attenuated, whereas a decrease in eye lens GPx activity was especially correlated with the severity of retinopathy (Livingstone 2007). There is an outstanding result that the GSH infusion of type 2 diabetes improved insulin sensitivity and increased the GSH/GSSG ratio in erythrocytes (De Mattia 1998; Franco *et al.* 2007). OxS contributes to the pathogenesis of chronic obstructive pulmonary disease (COPT) and several clinical trials are focused on testing the beneficial effects of antioxidants (Rahman 2008; Repine *et al.* 1997). COPT is also related with alterations in the glutathione system; for example, reduced erythrocyte GPx activity was correlated with the severity of COPT (Kluchova *et al.* 2007).

The participation of high-grade OxS in a number of pathological events creates the requirement for new molecules with promising antioxidative effects. At the same time, the exact role of OxS in the concrete pathogenetic mechanism of a disease must be specified to find the right targets and precise application ways/forms for antioxidant adjuvant therapy.

3.4 Glutathione analogues

Due to the versatile role of GSH, different strategies have been applied to maintain both the levels and functionality of the glutathione system. One of the

research objectives concerns how to restore the intracellular GSH level that may be potentially useful in pathological states related to GSH pool depletion. Reaching the sufficient cellular GSH concentration (up to some mM) by administering GSH itself is highly complicated, because of its rapid degradation in the digestive system and the difficulties with direct uptake into different cell types. The bioavailability of cysteine has been determined as the main limiting factor of the *de novo* synthesis of GSH. As the application of high doses of cysteine causes toxicity problems (Olney *et al.* 1990), cysteine precursors – for example, N-acetylcysteine – have been used (Bernard 1991; Ortolani *et al.* 2000). Due to the versatile roles of GSH, a number of GSH-like substances with extremely different properties have been synthesized (Lucente *et al.* 1998; Zilmer *et al.* 2005).

Various modifications in the structure of the GSH molecule have been performed to improve its stability and cellular uptake. The esterification of glycine with ethyl or isopropyl moiety yields in glutathione monoesters which are taken up by cells and hydrolysed to GSH is often used. Diesters (the esterification of the Gly and Glu α-carboxyl group) are proposed to be transported even faster into cells, where it rapidly splits to monoesters; but at the same time they are transported out of cells quickly as well. In addition, it is problematic to test diesters on rodents (except hamsters), as they have remarkably higher plasma diesterase activity compared to humans (Anderson 1998). Glutathione monoesters (ethylester is mostly used) have shown protective properties in models of stroke and spinal cord injury, PD, diabetic cataract, LDL oxidative modification and liver perfusion injury with rats (Anderson *et al.* 2004; Grattagliano *et al.* 1999; Guizar-Sahagun *et al.* 2005; Rajasekaran *et al.* 2005; Zeevalk *et al.* 2007; Zhang *et al.* 2008). GSH monoethyl ester protects against GSH deficiency due to biological aging and an acetaminophen-induced decrease of GSH pool in old mice (Chen *et al.* 2000). γ-glutamylcysteine ethylester is also used for neuroprotection in animal models (Chinta *et al.* 2006; Reed *et al.* 2009).

The GSH analogue YM737 [N-(N-r-L-glutamyl-L-cysteinyl) glycine 1-isopropyl ester sulfate monohydrate] has been shown to have protective qualities in rats' cerebral ischemia by inhibiting lipid peroxidation (Yamamoto *et al.* 1993).

In some clinical situations like cancer therapies, diminishing the GSH level in the cancer cell is the goal. The over-expression of GST has been reported to be one of the responsible biochemical mechanisms of drug resistance in cancer cells. GST plays an important role in the deactivation of a number of alkylating agents used in cancer therapies (Wu *et al.* 2004). In this way, a large number of GSH analogues have been designed to inhibit different GST isoenzymes: the phosphono-analogues (Kunze and Heps 2000) and the peptidometic analogues that are stable towards GGT, the main enzyme of GSH breakdown (Burg *et al.* 2002). One of the latest and more successful GSH analogues in cancer therapy, TLK 286, is in clinical trials (Rosen *et al.* 2003). Some designed GSH analogues act as glyoxalase inhibitors and have shown potent anti-proliferative and anti-tumour activity (Lo and Thornalley 1992a). The substitution of the

amino group at the GSH molecule N-terminus with a pyrrole ring gives new antioxidants that, due to steric hindrance, do not inhibit the GR nor the GPx (Gaullier *et al.* 1994). Replacing the native γ-glutamyl moiety with the *cis*- or *trans*-4-carboxyl-L-proline residue gives a conformationally rigid skeleton and makes this GSH analogue resistant to degradation by GGT (Paradisi *et al.* 2003). Still, the improvement of GSH analogues' stability towards enzyme activities (peptidases, GGT) stands as a general problem. One possible solution for overcoming this problem is through the cyclization of the GSH molecule. Such analogues have been tested for anti-tumor activity (Sheh *et al.* 1990). The special group of GSH analogues are cysteine-substituted S-nitrosoglutathiones that have been investigated based on the physiological roles of both GSH and NO (Richardson and Benjamin 2002). Table 1 describes an overview about different chemical modifications in the GSH molecule (Zilmer *et al.* 2005).

Considering the importance of glutathione, the maintenance of its adequate level, its decrease under different pathological states and the need for effective non-toxic glutathione analogues, the current work is focused on the creation and investigation of novel glutathione-like molecules, which may have the possibility of maintaining or improving the body defences under the condition of high-grade OxS, and will not impede the organism ADS.

γ-Glu-Cys-Z	
Z	Reference
-Gly-OAlk	(Exner *et al.* 2000)
YM 737 (L-glutamyl-L-cysteinyl-glycine 1-isopropyl ester)	(Yamamoto *et al.* 1993)
-Val, -Asp, -Phe, -GABA, -Lys, -His, -Ala, -D-Ala, -β-Ala, -PhGly	(Adang *et al.* 1990)
Cyclo(-γ-Glu-Cys-β-Ala-)	(Sheh *et al.* 1990)
Cyclo(-γ-Glu-Cys-GABA-)	(Sheh *et al.* 1990)
-Cys (Cys-Cys cycle)	(Calcagni *et al.* 1999)
γ-Glu-Y-Gly	
Y	Reference
S-alkyl-, S-aryl-, S-acyl-Cys	(Janaky *et al.* 2000)
β-Cl-Ala, 1-aminocyclopropane-1 carboxylic acid	(Morera *et al.* 2000)
S-(phenyl-thiosulfonyl)-Cys	(Adang *et al.* 1990)
Ala, Ser	(Krezel and Bal 2003) (Chen *et al.* 1985)
O-acetyl-, O-propionyl-, O-buturyl-, O-valeryl-Ser	(Lo and Thornalley 1992b)
Phosphono-Cys	(Kunze 1996)

S-nitroso-Cys	(Richardson and Benjamin 2002) (Janaky *et al.* 2000)
4-amino-1,2-dithiolane-4-carboxylic acid	(Morera *et al.* 2000)
β-methyl-cysteine	(Xie and Creighton 1991)
β,β-dimethyl-cysteine	(Adang *et al.* 1990)
X-Cys-Gly	
X	Reference
Phe-, Trp-	(Jaouhari *et al.* 1995)
Phosphonic analogue of γ-Glu	(Witkowska and Wasielewski 1989)
N-(2,5-disubstituted pyrrole)-Glu	(Gaullier *et al.* 1994)
Cis- or trans-4-carboxy-L-Pro-	(Paradisi *et al.* 2003)
X-Y-Z	
Substitutions at more than one site	Reference
TLK 286 ({L-γ-glutamyl-3-[[2-[[bis[bis(2-chloroethyl)amino]-phosphinyl]oxy]ethyl]sulfonyl]-L-alanyl-2-phenyl-(2R)glycine)	(Ruscoe *et al.* 2001) (Gate *et al.* 2003)
TLK 199 (gamma-glutamyl-S-(benzyl)cysteinyl-R-phenyl glycine diethyl ester)	(Rosen *et al.* 2003)
γ-(L-γ-azaglutamyl)-S-(p-bromobenzyl)-L-cysteinyl-glycine	(Vince *et al.* 1999)

AIMS OF THE STUDY

The general aim of the present study was to create a library of novel glutathione analogues (UPF peptides) and their primary characterisation as potential anti-oxidative protector-molecules.

The concrete aims of the study were defined as follows:

1. To design and synthesise a novel peptidic glutathione analogues library (UPF peptides library).
2. To measure the free radical scavenging ability of members of this library and to select from these at least two molecules with physiologically relevant antioxidative properties for further investigation.
3. To investigate the stability of selected members of the UPF peptides in water, physiological solution, phosphate buffer, H_2O_2 and $CuSO_4$ solutions and assess the toxicity of these UPF peptides on human erythroleucemia K562 cells.
4. To study the effects of selected members of the UPF peptides library on the activities of two antagonistic targets: a) antioxidative enzymes (GPx, GR, MnSOD), and b) potentially pro-oxidative superoxide producing enzyme NAD(P)H oxidase.

MATERIALS AND METHODS

1. Peptide synthesis

1.1 Design of peptides

Various low molecular weight antioxidants, including melatonin, carvedilol and its metabolite SB 211475, carry a methoxy moiety in their aromatic structures. Gozzo *et al.* modified different parts in melatonin structure to clarify the structural features responsible for its antioxidant activity (Gozzo *et al.* 1999). They tested the antioxidative properties on low-density lipoprotein oxidation model and found that the compounds with methoxy group showed higher antioxidant activity than their analogues lacking this group. The replacement of methoxy moiety with hydroxyl group increased antioxidativity even more but some experimental models have proposed the pro-oxidant activity of phenolic compounds. With appropriate side chain, some methoxy derivates were equally active. Antihypertensive drug carvedilol and its analogue SB 211475 have both methoxy moiety connected with aromatic ring whereas SB 211475 possess even higher antioxidative properties assessed in a variety of models including superoxide release from phorbol ester stimulated human neutrophils (Yue *et al.* 1994). SB 211475 is the main metabolite of carvedilol and the only difference is that OH group is introduced at the aromatic structure.

Gathering this information together with analysis of impressive biofunctionality of glutathione, first the tetrapeptide UPF1 was designed, where *O*-methyl-L-tyrosine was added to the N-terminus of glutathione. Further we designed and prepared a series of UPF peptides (Paper I) to improve the quality of protective properties and investigate the structure-based effects. Design strategy was to prepare the analogues not largely different by structure compared with glutathione, to keep their toxicity as low as possible. Various amino acids were added to a glutathione molecule via a peptide bond, resulting in tetrapeptide library. Considering the importance of hydroxyl group in antioxidant activity, analogues with tyrosine (UPF15) or with serine (UPF6, UPF16, UPF19, UPF24, UPF27) were synthesized although the *O*-methyl-L-tyrosine was most used in different sequences. Mainly the additional unit was added to the N-terminus, but positioning in the C-terminus was also investigated (UPF2) to study interactions of methoxy group and free C-terminal carboxyl group. Part of the library was synthesized so that α-glutamyl moiety was used instead of γ-glutamyl moiety (UPF17–UPF25). In the case of several peptides, all L-amino acids (UPF10, UPF14) or only the first N-terminal L-amino acid were substituted with their D-analogues (UPF5, UPF6, UPF18, UPF19 and UPF27) to both control stereoisomeric impact on antioxidative properties and to improve their resistance towards endogenous peptidases. The amidation of the C-terminus (UPF8, UPF10, UPF25 and UPF27) was used with the same purpose. UPF26 was the only peptide where cysteine was replaced with serine to observe

the influence caused by the removal of the thiol group. This work is based on aforementioned 17 glutathione analogues and concerning more targeted information on selected members of UPF peptide library. The design and synthesis of UPF peptides is further carried on.

1.2 Synthesis and purification of peptides

Nowadays, solid phase peptide synthesis (SPPS) is a routine method for peptide synthesis. At first, SPPS was introduced by Bruce Merrifield in 1963 and in 1984 it was awarded the Nobel Prize in Chemistry. The principle of SPPS is very simple: during the synthesis the peptide is covalently attached to an insoluble carrier called resin, which is stable towards the chemical reactions of peptide synthesis. SPPS has several advantages compared to peptide synthesis in solution. Attachment to the solid support reduces aggregation and enables the usage of high reagent quantities which can be easily washed away after each step is completed. In addition, there is no need for intervening separation of side products. Amino acids are assembled together in a stepwise manner by repeated formation of peptide bonds. Two main types of SPPS exist, depending on the temporary N-terminal protecting groups chemistry: first, acid-labile *tert*-butyloxycarbonyl group (*t*-Boc-group) or secondly, base-labile 9-fluorenylmethoxycarbonyl group (Fmoc-group). Reactive groups of side chains are blocked by permanent protective groups, which are removed together with release of the peptide from the resin, called cleavage, after the synthesis is completed. The synthesis is usually and also in current work carried out from the C-terminus to the N-terminus of the peptide so the carboxyl group of new amino acid will form the peptide bound with amino group of the amino acid already attached to the resin. For this, previous deprotection of amino group and activation of carboxylic acid group are required.

The glutathione analogues were synthesized manually using Fmoc-chemistry (Soomets *et al.* 2005). Fmoc-method was preferred because of safety as it has milder cleavage conditions – trifluoroacetic acid (TFA) compared to the superstrong acid HF in the *t*-Boc-method. Most of the UPF peptides have glycine as C-terminal amino acid so the Gly-Wang resin with commercially pre-attached Gly was used. Couplings of Fmoc protected amino acids were carried out in a stepwise manner using the standard carboxylic acid group activation by 2-(1H-bensotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) and 1-hydroxybenzotriazole (HOBt) in dimethylformamide (DMF). N,N-diisopropylethylamine (DIEA) was added for *in situ* neutralization. Piperidine was used for Fmoc group removal. The peptides were detached from the resin and simultaneously deprotected with TFA in the presence of scavengers (prevent the reactions with side chains of the peptides), water 2% (v/v), EDT 2% (v/v) and TIS 2.5% (v/v) for 90 minutes at room temperature. To collect the synthesis product, the resin was removed by filtration under the reduced pressure. TFA and scavengers were evaporated by using a rotating evaporator. The product

was washed with cold ether repeatedly and after final centrifugation the pellet was dissolved in 20% acetonitrile/water mixture and lyophilized.

Reverse-phase high performance liquid chromatography (RP-HPLC) is a routine method for peptide purification based on the hydrophobic interactions between a non-polar stationary phase and an analyte in an aqueous, moderately polar mobile phase. The purity of UPF peptides was > 99% as demonstrated by HPLC on an analytical Nucleosil 120-3 C18 reversed-phase column (0.4 cm × 10 cm). Routinely the crude peptides were purified by the semi-preparative RP-HPLC column (ZORBAX 300 SB-C18 9.4 mm × 25 cm, a 1100 Hewlett Packard HPLC apparatus) employing an acetonitrile/water mixture (containing 0.1% TFA) as an eluent at a flow rate of 4 ml/min and absorbance of 218 nm. Purified peptides were lyophilized.

The molecular masses of the peptides were determined by a matrix-assisted laser desorption ionization time-of-flight mass-spectrometry (MALDI-TOF MS) (Voyager DE Pro, Applied Biosystems) and the calculated values were obtained in each case. According to the Applied Biosystems guidelines, saturated solution of α-cyano-4-hydroxycinnamic acid was chosen for the matrix (10 mg/ml in 50:50 acetonitrile/water mixture with 0.1% TFA content). Dried droplet method was used for the sample preparation – the peptide solution and the matrix were mixed together on the sample plate and air dried.

2. Stability analysis of UPF peptides

2.1 Estimation of the dimerization of UPF peptides by RP-HPLC

Dimerization over the disulphide bridge is a key issue in the stability of low molecular weight thiols if stored as solutions. The dimerization rate of UPF1 and UPF17 was estimated by RP-HPLC in water and in physiological solution (0.9% NaCl). UPF1 and UPF17 were chosen as the representatives of UPF peptides with the two different glutathione backbones containing γ- and α-glutamate residue, respectively. 1 mM solutions of peptides were kept at room temperature and at certain time points during 14 days, samples were taken and analyzed on analytical RP-HPLC column (ZORBAX 300 SB-C18 4.6 mm × 15 cm) using a linear acetonitrile-water gradient from 20% to 90% acetonitrile (v/v) (0.1% TFA) at a flow rate 2 ml/min. The wavelength of peak detection was 220 nm. Fractions were collected and molecular masses of peptides (monomeric and dimeric forms) were determined by the MALDI-TOF MS. In addition, for UPF1 and UPF17 the peak areas were calculated by the ChemStation software of Hewlett Packard HPLC system (model 1100). The quantities of the reduced and oxidized forms of studied analogues in the sample were expressed in percents. Summarized areas under the peaks of monomeric and dimeric forms of UPF peptides were constant and were considered as 100% through all experiments.

2.2 Estimation of the dimerization of UPF peptides by capillary electrophoresis

Selected UPF peptides were applied for capillary electrophoresis (CE) analysis to characterize the dimerization and reactivity towards H_2O_2 and Cu^{2+} ions. Concentrations of GSH and GSSG are usually determined by fluorimetric assay or HPLC methods. Besides HPLC, CE has become a powerful tool for the analysis of polar peptides and has been successfully applied for the separation of closely related peptides such as monomeric and dimeric forms of glutathione in biological systems and to monitor the kinetics of oxidation (Maeso *et al.* 2005). An analyte separation in CE is based on the formation of distinct migrating zones when an electric field is applied on narrow-bore capillary. Compared to the HPLC, CE technique offers significant advantages such as a high speed of separation and low sample and buffer requirements.

Capillary electrophoresis analyses were performed using an in-house built (laboratory-built) CE system, which was equipped with fused silica capillary (Polymicro Technologies, Phoenix, AZ USA), 55 cm (effective length 39 cm) × 50 μm I.D., high voltage power supply (Spellmann, Hauppauge, NY, USA) and UV detector (Prince Technologies). The UV detector was coupled to a personal computer, data acquisition was done by software written in-house, using LabView program (National Instruments, Austin, TX, USA). The software recorded the detector signal via ADAM 4018/4060 interface (Advantech Inc., Taipei, Taiwan).

The separation of peptides was performed in 25 mM phosphate buffer (pH 7.5) containing 50 mM sodium dodecyl sulphate (SDS) using the applied voltage of 25 kV at 22 °C. This type of CE is called micellar electrokinetic chromatography and its advantage is improved separation efficiency. Under these conditions the peptides separation was achieved due to different charge-to-mass ratio and to hydrophobic interaction with SDS micelles. The detection was performed at 215 nm, according to the absorbtion maximum for peptide bond. The peaks in electropherogram must be identified by the standard adding method. Standards and samples were injected into the capillary gravitationally during a fixed time of 10 s. Capillary was conditioned prior to use with 1 M NaOH for 20 min and with H_2O for 30 min. After each run capillary surface was regenerated by sequential washing with 0.1 M NaOH, H_2O and separation buffer for 5 min each.

The peptides GSH, UPF1, UPF6, UPF17, UPF19 (250 μM) were oxidized with H_2O_2 (1 mM) and their oxidation kinetics were observed (Paper II). The oxidation process of GSH, UPF1 and UPF15 (250 μM) were monitored in the 15 mM phosphate buffer (pH 7.5), copper(II)sulphate (at 20 μM) or hydrogen peroxide solutions (at 1 mM) until the disappearance of a monomeric form of peptides established by CE (Paper III).

In addition, the oxidation reaction of the mixture of GSH and UPF1 (250 μM each) in the presence of 1mM H_2O_2 was followed by CE in both papers (Paper II, Paper III). MALDI-TOF analysis was also used for detecting the heterodimer

formation between GSH and UPF1 (250 μM peptides, 1mM H_2O). Before analysis, the mixture of peptides was stirred for 1.5 hours at room temperature to complete the reaction and reduce the oxidizing activity of H_2O_2 towards the matrix. The sample preparation technique was the same than described above in UPF purity control analysis.

3. Free radical scavenging assays

3.1 Hydroxyl radical scavenging assays

The hydroxyl radical scavenging ability of UPF peptides was measured by using terephthalic acid (benzene-1-4-dicarboxylic acid, THA) as a chemical dosimeter (Barreto *et al.* 1995). The advantage of THA compared to benzoate and salicylate dosimeters is that due to the symmetry of molecule, THA forms only one strongly fluorescent product with hydroxyl radical. Similarly to benzoate, the target THA reactant is non-fluorescent. Although the direct way to measure radical formation is electron spin resonance method, in practice this approach is limited by the expense and availability of the equipment for routine work. The final concentration of THA was 10 mM and hydroxyl radical was generated via Fenton-like reaction between $CuSO_4$ and H_2O_2 with final concentrations of 10 μM and 1 mM, respectively. All the solutions used were prepared in 14.75 mM sodium phosphate buffer at pH 7.5. The hydroxyl radical suppression was measured by a spectrofluorescence method at 312 nm excitation and at 426 nm emission (Perkin-Elmer LS50B). The hydroxyl radical elimination was expressed in EC_{50} values determined by sigmoid dose-response (viable slope) analysis.

Alternatively the rate of the scavenging capacity of the hydroxyl radical was monitored by CE assay for selected peptides. In this case, hydroxyl radical elimination was estimated based on concomitant dimerization of UPF peptides. For 250 μM GSH, UPF1, UPF6, UPF15, UPF17, UPF19 the hydroxyl radical-generating system consisted of copper(II)sulphate (10 μM) and of H_2O_2 (1 mM), both in 15 mM sodium phosphate buffer (pH 7.5). Details of the formed compound separation by CE method are described in chapter 2.2.

3.2 DPPH radical scavenging assay

This method, α,α-diphenyl-β-picrylhydrazyl radical ($DPPH^{\bullet}$) scavenging ability, is widely accepted for evaluation of antioxidant activities of different compounds (Fagali and Catala 2008; Jimenez-Escrig *et al.* 2000). DPPH radical is stable and has high steric hindrance due to three aromatic rings (Figure 8). The scavenging effect of DPPH radical was measured spectrophotometrically (Jenway 6405 UV/Vis spectrophotometer, Jenway Ltd.). UPF peptide solution

in 0.9% NaCl at a concentration range from 2.5 to 200 μM was added to 0.1 mM DPPH$^{\bullet}$ in 95% ethanol. Absorbances at 517 nm were recorded from 0.5 min up to the time when a steady state was reached. A lower absorbance represented a higher DPPH$^{\bullet}$ scavenging activity. The percentage of remaining DPPH$^{\bullet}$ against the peptide concentration was plotted to obtain the amount of antioxidant necessary to decrease by 50% the initial DPPH$^{\bullet}$ concentration (EC_{50}). The time needed to reach the steady state to EC_{50} concentration (T_{EC50}) was calculated graphically. The scavenging effect was expressed as antiradical efficiency (AE). AE is $1/EC_{50}T_{EC50}$ (Sanchez-Moreno *et al.* 1998).

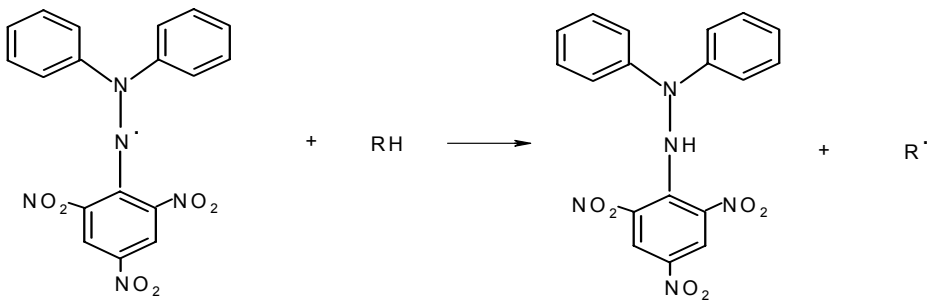

Figure 8. Structures of DPPH radical and non-radical forms. RH is a proton donating antioxidant.

4. Toxicity tests of UPF peptides

After measurements of the antioxidative potential of UPF peptides, an initial test on a cell culture was needed to estimate the toxicity of most promising analogues (UPF1, UPF6, UPF17 and UPF19) with different GSH backbone. Lactate dehydrogenase (LDH) release and ATP production were chosen as quantifiable markers of cell viability, which are also successfully used in the toxicity test of cell penetrating peptides (Saar *et al.* 2005). The effects of UPF peptides on blood cells were already studied, thus K562 human erythroleukemia cells were chosen for the cell culture. Although often used in cancer and differentiation studies, K562 cells are also used for the antioxidant toxicity evaluations (Mohammadi and Yazdanparast 2009).

K562 human erythroleukemia cells were propagated in suspension using RPMI-1640 medium supplemented with GlutaMAX™-I, penicillin (100 units/ml), streptomycin (100 μg/ml) and heat-inactivated foetal bovine serum (7.5%). Cell density was kept between 10^5–10^6 cells/ml. The cells were cultured at 37 °C in 5% CO_2. The plastic labware (Corning®) was from Labdesign AB (Täby, Sweden) and cell culture reagents (GIBCO™) from Invitrogen AB (Lidingö, Sweden).

CellTiter-GloTM Luminescent Cell Viability Assay (Promega, Madison, WI) was used to estimate the effects of UPF1, UPF6, UPF17, and UPF19 on K562 cell viability via the quantification of ATP production. K562 cells were suspended into wells of a 48-well-plate. The final concentrations were 200 µM for peptides (UPF1, UPF6, UPF17, UPF19) and 20 µM for methotrexate (MTX), which was used as positive control. After 24 h of exposure in the cell culture incubator, the plate was equilibrated to room temperature for approximately 30 min and the CellTiter-Glo™ Reagent was added. After 10 min the luminescence was recorded on a dual-scanning microplate spectrofluorometer SPECTRAmax® GEMINI XS from Molecular Devices (Sunnyvale, CA) on a white polypropylene LumiNunc™ plate (Nunc A/S, Roskilde, Denmark).

CytoTox-One™ Homogenous Membrane Integrity Assay (Promega, Madison, WI) was used to estimate the effects of UPF1, UPF6, UPF17 and UPF19 on the membrane integrity of K562 cells. This assay is based on the measurement of LDH release from cells with a damaged membrane. Cells ($\leq 10^6$ cells/ml) were centrifuged for 5 min at 500*g* and the cell pellet was washed twice with 10 ml HEPES Krebs Ringer solution (5.5 mM HEPES, 138 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5.6 mM glucose, pH 7.4). The cells were then resuspended at a density of 1 x 10^6 cells/ml (counted with hemacytometer). Part of this suspension was transferred to a vial which already contained an equal volume of peptide or Triton X-100 in HEPES Krebs Ringer solution. The final concentrations were as follows: 100 µM for UPFs, 10 µM for model amphipathic peptide (MAP) and 0.1% for Triton X-100. After 10 min of incubation at 37 °C at 300 rpm in a Thermomixer (Eppendorf AG, Hamburg, Germany) the vials were centrifuged for 2 min at 500*g*. Then 100 µl of the supernatant was transferred to a black polypropylene FluoroNunc™ plate (Nunc A/S, Roskilde, Denmark) and CytoTox-One™ Reagent was added to each well. After 10 min of incubation at room temperature, stop solution was added and the fluorescence was recorded on a dual-scanning microplate spectrofluorometer SPECTRAmax® GEMINI XS from Molecular Devices (Sunnyvale, CA) using following wavelengths: 560 nm excitation and 590 nm emission.

5. Measurements of enzyme activities

5.1 Assays of GPx and GR activities

The GPx and GR activities are responsible for peroxide elimination by GSH and further GSSG conversion back to the reduced form, respectively. UPF peptides as glutathione analogues may be potential inhibitors of these vital enzymes. Thus, their influence on these enzymes must be tested. The sources of GPx were: first, commercial purified enzyme from bovine erythrocytes (obtained form Sigma-Aldrich, Germany) and second, erythrocyte lysate.

Erythrocyte separation and measurement of GPx activity were performed according to the Bioxytech GPx-340 Assay kit (Bioxytech®, OXIS International, Inc., Portland, USA) manual. The reaction mixture of the assay kit contained glutathione, glutathione reductase and NADPH. GPx converts GSH to GSSG, which is immediately converted back to the reduced form by the GR with a concomitant oxidation of NADPH to $NADP^+$. Accompanied decrease in absorbance of NADPH at 340 nm was measured spectrophotometrically on a Microtiter Plate ELISA Reader. To measure the concentration dependent influence of UPF1 and UPF17 on GPx activity, the solutions of those peptides were added to the reaction mixture with final concentrations of 25–1500 μM. After 5 minutes preincubation time for peptides, tert-butyl hydroperoxide was added to initiate the enzyme reaction. The change of NADPH concentration was recorded for 3 minutes with taking a reading every 30 seconds. GPx activity is expressed as relative to basal activity (100%) of GPx.

In erythrocyte lysate, kinetics of inhibition was also followed. The effect of UPF17 was studied as it showed more expressed inhibition of GPx activity compared to UPF1. Peptides were used in following concentrations: GSH as substrate for GPx with concentrations of 0.5, 1.0, 1.5 mM and UPF17 as inhibitor with concentrations of 0.1 and 0.15 mM. In order to confirm the type of inhibition of GPx by UPF17, the Lineweaver-Burk plot was constructed.

Also the activity of GR (from baker's yeast; obtained from Sigma-Aldrich, Germany) was measured spectrophotometrically according to the Bioxytech GR-340 Assay (Bioxytech®, OXIS International, Inc., Portland, USA). Its activity was determined by the time dependent change in the added NADPH concentration. Final enzyme concentration in the assay was 8.4 mU/mL. The final concentrations of GSH, UPF1 and UPF17 were from 0.2 to 1.0 mM. Preincubation time was 5 minutes and 24 h for peptides. GR activity is expressed as relative to the basal activity (100%) of GR.

5.2 MnSOD activity in brain cortex mitochondria

There are different types of superoxide dismutases but the purpose of this research was to study mitochondrial MnSOD from brain tissue as UPF1 has shown some neuroprotective qualities in global brain ischemia model in rats (Põder *et al.* 2004). Human brain tissue was obtained from the Huddinge Brain Bank, Huddinge University hospital, Sweden. The mitochondria were isolated from post mortem human temporal cortex (aged 86–92 years) via centrifugation on Percoll density gradient according to Rajapakse *et al.* (Rajapakse *et al.* 2001). Intact mitochondria were incubated with GSH at concentration 10 nmol/mg protein during 5 and 40 minutes. UPF1 and UPF17 were used at concentrations 0.5, 1.0 and 5.0 nmol/mg protein with the incubation times 5 and 40 minutes. The protein content in the enzyme preparations was determined by the method of Lowry (Lowry *et al.* 1951). MnSOD activity was measured with the commercially available Ransod kit (Randox Laboratories Ltd. Ardmore,

UK). Reaction between xanthine and xanthine oxidase is used for superoxide radical generation, which further forms a red formazan dye with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium (I.N.T). The MnSOD activity was assessed as a degree of inhibition of this reaction. In the content of mitochondrial fraction added MnSOD will compete with I.N.T resulting in spectrophotometrically detectable reduced formation of formazan dye. To inhibit possible Cu,ZnSOD activity, 5 mM KCN was added to the reaction mixture.

5.3 Measurement of superoxide radical generation in isolated human PMNs

The NAD(P)H oxidases are the main sources of superoxide radical in neutrophils and vascular tissues and thus contribute to also abnormal inflammatory response. Thereby, novel NAD(P)H oxidase inhibitors are searched and investigated as potential anti-inflammatory substances (Van den Worm *et al.* 2001). Influence of UPF peptides on superoxide radical generation in isolated human blood polymorphonuclear neutrophils (PMNs) was assessed by chemiluminescence method using luminol analogue (8-amino-5-chloro-7-phenylpyrido[3,4-d]pyridazine-1,4-(2H,3H)dione sodium salt (L-012) as a dye. L-012 has shown to be highly sensitive and specific towards superoxide radical, whereas traditionally used chemiluminescence dyes have some drawbacks (Daiber *et al.* 2004). Luminol for example lacks specificity for superoxide and at higher concentrations lucigenin tends to show superoxide radical production higher than actual.

Venous blood with EDTA supplement was obtained freshly from volunteered blood donors of local donor centre. RBCs were separated by sedimentation with methylcellulose-25. White blood cells fraction containing supernatant was diluted with Ca^{2+} and Mg^{2+} free Hank's Balanced Salt Solution (HBSS) and centrifuged on Ficoll-Paque Plus density gradient (Amersham Biosciences AB, Uppsala, Sweden) for 30 min at 400*g* and 20 ºC. The remaining erythrocytes were removed by hypotonic lysis. PMNs pellet was resuspended in HBSS and counted automatically with Sysmex-XE2100. The neutrophil count in reaction mixture was kept between 60 000–160 000 cells. Concentrations for UPF peptides were 10 and 100 μM and 200 μM for L-012. After 3 minutes of incubation at 37 ºC in the presence of UPF peptides followed by 10 minutes of dark adaption, the superoxide burst was initiated using 1 μM phorbol 12,13-dibutyrate (PDBu; obtained from Sigma-Aldrich, Germany) as a NAD(P)H oxidase activator. Validation of this assay has been reported previously (Daiber *et al*. 2004; Mollnau *et al*. 2003).

6. Other methods

Influence of UPF1 and UPF17 on GPx and MnSOD expression was studied by RT-PCR analysis. Human blood mononuclear cells were separated using BD Vacutainer® CPT tubes (Becton Dickinson, Franklin Lakes, NJ, USA) The mononuclear cells were incubated 12 hours in the presence of 0.5 μM UPF1 or 0.5 μM UPF17 using RPMI-1640 medium (includes 10% fetal calf serum and 1% penicillin⁄streptomycin). RNA was extracted from the mononuclear cells with the Trizol method and cDNA was synthesized according to the manufacturer's protocol (Invitrogen, San Diego, CA, USA.). Gene expression levels were detected using the TaqMan-QRT-PCR method (ABI Prism 7900HT Sequence Detection System, Applied Biosystems, Foster City, CA, USA). For quantification of mRNA comparative Ct method (ΔCt value) was used, where the amount of target transcript was normalized according to the level of endogenous reference HPRT-1 (hypoxanthine phosphoribosyl-transferase-1).

Circular dichroism spectroscopic (CD) measurements were performed in Stockholm University to investigate if UPF peptides (UPF1, UPF6, UPF17, UPF19) can form aggregates or any secondary structures in water solution.

7. Statistics

Data were analyzed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA, USA). Reaction rate constants are given with SD. For statistic analysis of PCR results, Mann-Whitney U test was used. Other results are presented as the mean values ± standard error of the mean (SEM) and compared with the paired or unpaired Student's t-test respectively. Significance was defined as $P < 0.05$.

RESULTS

1. Peptide design and synthesis (Paper I)

The library of glutathione analogues, called UPF peptides, was designed and synthesized (Paper I). Compared to the GSH structure, the following changes were made in the sequence of novel analogues: the addition of the fourth amino acid to the N- or C-terminus of the GSH backbone, the replacement of the γ-glutamyl moiety with the α-glutamyl moiety, a change of chirality (L- to D-amino acids) and the amidation of the terminal carboxylic acid group. All the synthesized peptides were soluble in water solutions up to 1 mM, except the biotinylated analogue, UPF7. UPF7 was designed to study the intracellular location of UPF1 by fluorescence methods, but hydrophobic biotinyl moiety significantly decreased the solubility of the analogue in water solutions, prohibiting the use of it in the following experiments.

Table 1. Library of designed and synthesized glutathione analogues – UPF peptides.

Nr.	Sequence	MW
UPF1	H_2N-Tyr(Me)-(γ-Glu)-Cys-Gly-COOH	484.5
UPF2	H_2N-(γ-Glu)-Cys-Gly-Tyr(Me)-COOH	484.5
UPF5	H_2N-D-Asp-(γ-Glu)-Cys-Gly-COOH	421.4
UPF6	H_2N-D-Ser-(γ-Glu)-Cys-Gly-COOH	394.4
UPF7	Biotinyl-Tyr(Me)-(γ-Glu)-Cys-Gly-COOH	710.5
UPF8	H_2N-Tyr(Me)-(γ-Glu)-Cys-Gly-$CONH_2$	483.5
UPF10	H_2N-D-Tyr(Me)-D-(γ-Glu)-D-Cys-Gly-$CONH_2$	483.5
UPF14	H_2N-D-Tyr(Me)-D-(γ-Glu)-D-Cys-Gly-COOH	484.5
UPF15	H_2N-Tyr-(γ-Glu)-Cys-Gly-COOH	469.4
UPF16	H_2N-Ser-(γ-Glu)-Cys-Gly-COOH	394.4
UPF17	H_2N-Tyr(Me)-Glu-Cys-Gly-COOH	484.5
UPF18	H_2N-D-Tyr(Me)-Glu-Cys-Gly-COOH	484.5
UPF19	H_2N-D-Ser-Glu-Cys-Gly-COOH	394.4
UPF24	H_2N-Ser-Glu-Cys-Gly-COOH	394.4
UPF25	H_2N-Tyr(Me)-Glu-Cys-Gly-$CONH_2$	483.5
UPF26	H_2N-Tyr(Me)-(γ-Glu)-Ser-Gly-COOH	468.5
UPF27	H_2N-D-Ser-(γ-Glu)-Cys-Gly-$CONH_2$	393.4

2. Stability of UPF peptides

2.1 Stability in water and physiological solutions characterized by RP-HPLC (Paper I)

As in the water solutions glutathione dimerization occurs, the rate of dimer formation of the preselected UPF peptides (UPF1, UPF17) in water and in a physiological solution was studied to clarify the state of the molecules concerning the following experiments (Figure 9). In the water and physiological solution, analogues showed a different dimerization rate at 1 mM concentration (Paper I). For all of the peptides studied, the dimerization rate was higher in the saline solution than in water. For example, after the 14th day 27.5% of UPF1 was dimerized in water, when in saline the same amount of UPF1 dimer was detected on the 4th day. On the 14th day UPF17 showed 17.9% of dimerization in the water solution, whereas in the saline it was detected on the 7th day.

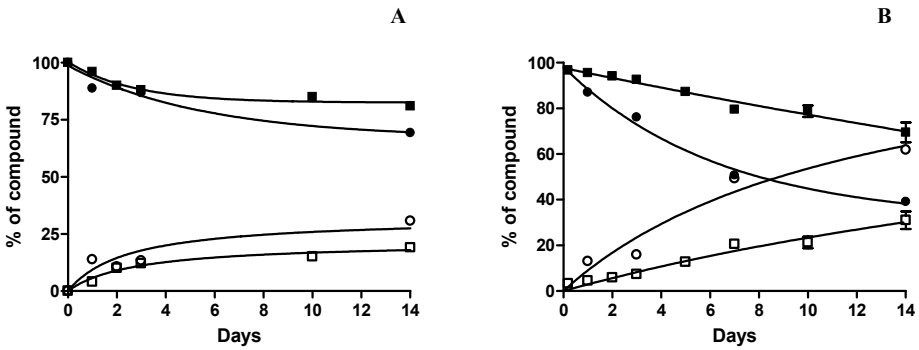

Figure 9. Dimerization of UPF1 and UPF17 in water and in 0.9% NaCl solution at room temperature. Content of monomeric (●) and dimeric (○) form of UPF1 and of monomeric (■) and dimeric (□) form of UPF17 in water (A) and in 0.9% NaCl solution (B).

2.2 Stability in different solutions characterized by capillary electrophoresis (Paper II, Paper III)

The changes in the composition of the mixture of GSH and selected UPF peptides (UPF1, UPF6, UPF17 or UPF19) in the presence of the oxidizing agent H_2O_2 were characterized by capillary electrophoresis (CE) (Paper II). The peaks of GSH and GSSG were identified by a standard adding method. Based on the analogy with GSH, the peak of the dimerized form of UPF peptides was determined and also followed in MALDI-TOF MS. The mixture with UPF1 is presented as an example (Paper II); in the case of UPF6, UPF17 and UPF19 analogous results were obtained. Beside monomeric and homodimeric forms of the GSH and UPF peptides, a remarkable quantity of an additional compound

was observed. This compound was supposed to be the heterodimer between GSH and UPF1, and the experiments with MALDI-TOF MS confirmed the essential formation of heterodimer (Paper II). The oxidation reaction between GSH and the respective UPF peptide mixture and hydrogen peroxide was also monitored at different time periods and was almost completed after 30 minutes at room temperature for all investigated peptides. Although in the MALDI-TOF mass spectras all homo- and heterodimers were indentified, MALDI-TOF MS is not a quantitative method and therefore CE was used to estimate the amount of reaction products. The CE method showed that the formation of heterodimer was prevailing and the production of mixed dimer was approximately two-fold higher compared to homodimers. At half-life time, the first- and second-order rate constants of the oxidizing reactions of peptides (UPF1, UPF6, UPF17, UPF19, GSH, GSH + UPF1, GSH + UPF6) with H_2O_2 were determined. The second rate-constants of the studied peptides were very close (for example, for GSH 0.204 and for UPF1 0.208 $1/M \times min \times 10^3$), showing that the structural features of peptides do not significantly influence the rate of the oxidizing reaction induced by H_2O_2 (Paper II). In the mixtures of the GSH and UPF peptides, the rate constants were also similar, indicating that the oxidation was not affected by the presence of the other peptide.

The stability of GSH, UPF1 and UPF15 were examined in different solutions: water, a physiological solution, a phosphate buffer, a copper(II)-sulphate solution and a hydrogen peroxide solution (Paper III). GSH was more stable in water and in the physiological solution, compared to UPF1 and UPF15, at room temperature. The oxidized form of GSH (GSSG) detected by MALDI-TOF MS appeared after 18 days of incubation, while the appearance of the disulphides of UPF1 and UPF15 was observed already after 2 days. In all cases, the oxidation in the phosphate buffer was considerably faster: the first signs of GSH dimerization appeared after 16 hours and for UPF1 and for UPF15 after 4 hours, detected by CE. Comparing the half-life times, the rate of dimerization in the solution of H_2O_2 was similar for all selected peptides (GSH 810 ± 48, UPF1 738 ± 31, UPF15 768 ± 31 s, respectively) (Paper III). There was also no difference in half-life time between the GSH/UPF1 mixture and the pure components in a hydrogen peroxide solution. The differences appeared in the $CuSO_4$ solution, where the formation of the dimers of the UPF peptides took place about two times faster than for the GSH dimer. Respective half-life times were as follows: GSH 10800 ± 520 s, UPF1 4200 ± 180 s, UPF15 4800 ± 210 s (Paper III). Comparing the different environments, in $CuSO_4$ the half-life time of GSH was approximately 10 times, and for UPF1 and UPF15 5–6 times longer than that in the H_2O_2 solution (Paper III).

3. Free radical scavenging studies

3.1 Hydroxyl radical scavenging assays (Paper I, Paper II, Paper III)

The hydroxyl radical scavenging assay was used as the first evaluation criteria of the antioxidativity of the designed peptides. The hydroxyl radicals were generated via a reaction between Cu^{2+} and H_2O_2 and detected by a fluorescence method using THA as a probe. The EC_{50} of the hydroxyl radical scavenging reaction for all water soluble UPF peptides in the library were found. The results showed that all designed and synthesized peptidic molecules were substantially stronger hydroxyl radical scavengers than GSH (Paper I). Peptides with a γ-peptide linkage in the backbone showed the EC_{50} between 17–35 μM compared to GSH 1231 μM, respectively (Figure 10). The substitution of all or only the N-terminal L-amino acid to D-isomer did not change the hydroxyl radical scavenging ability. The comparison of the radical scavenging properties of the free acid and amidated form in the next pairs of peptides – UPF1 and UPF8, UPF14 and UPF10, UPF6 and UPF27, UPF17 and UPF25 – revealed a tendency that peptide amides were slightly weaker hydroxyl radical scavengers than free acids. Surprisingly, the substitution of cysteine with serine residue in UPF26 sequence did not reduce the hydroxyl radical scavenging ability, suggesting that the thiol group is not the only one involved in hydroxyl radical scavenging activity. The most powerful antioxidants in the hydroxyl radical scavenging assay turned out to be the sequences where γ-glutamate residue was replaced with α-glutamate residue (UPF17–UPF25). These analogues showed the EC_{50} of the scavenging reaction in the submicromolar range (EC_{50} 30–50 nM), and the peptide solution with a concentration of 1 μM achieved approximately 80% of the maximal inhibiting effect (Figure 10). Very precise EC_{50} measurements in the case of α-glutamyl moiety containing peptides were disturbed by a slight elevation of the radical production in the end of the run if concentrations below 0.5 μM were used, probably caused by the extremely small amount of peptides.

Additionally, hydroxyl radical scavenging by GSH and selected UPF peptides (UPF1, UPF6, UPF17, UPF19) was observed by CE according to their disulphide formation rate via the reaction between two peptide molecules (Paper II). At half-life time, first- and second-order rate constants were determined. According to these results UPF1 and UPF6 showed higher antioxidative potential (respective second-order rate constants of 4.428 and 2.152) than UPF17, UPF19 and GSH (respective second-order rate constants of 0.99, 1.006 and 0.926). These different results correlate with the peptides' structures: UPF1 and UPF6 have the γ-glutamyl moiety in the backbone, while UPF17 and UPF19 contain the α-glutamyl moiety. In addition UPF1 was compared to its analogue UPF15, where methylated tyrosine is replaced by a tyrosine possessing hydroxyl group. In CE experiments in the presence of hydroxyl radicals, their half-life times were similar (41.4 ± 4.1 s for UPF1 and 42.6 ± 4.8 s for

UPF15) but showed significantly more radical scavenging potency than GSH (180.6 ± 8.3 s) (Paper III).

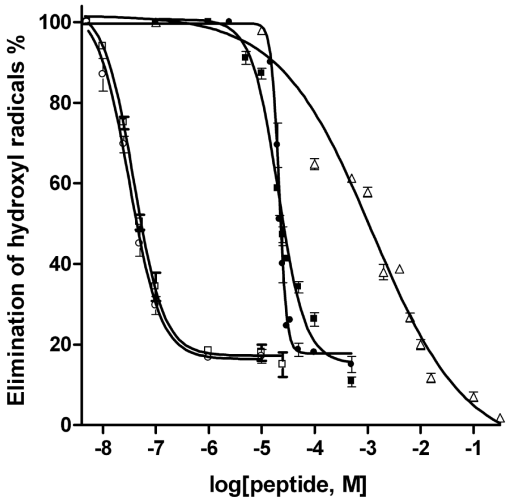

Figure 10. Concentration dependent hydroxyl radical scavenging effects of GSH (△), γ-glutamyl moiety containing UPF1 (■), UPF6 (●) and α-glutamyl moiety containing UPF17 (□), UPF19 (○) *in vitro*.

3.2 DPPH radical scavenging assay (Paper I)

This method is based on the reduction of DPPH$^{\bullet}$, a stable free radical. DPPH$^{\bullet}$ has a strong absorption at 517 nm, and this absorption is decreased upon reduction by a free radical scavenging antioxidant. The effects of selected UPF peptides were studied and the results showed that they all exhibited free radical scavenging activity against DPPH$^{\bullet}$. EC_{50} concentrations, the time needed to reach the steady state EC_{50} concentrations (T_{EC50}), and antiradical efficiency (AE, $1/EC_{50}T_{EC50}$) were found. Compared to GSH all designed molecules have similar, approximately 1.2-fold, higher EC_{50} concentrations required to scavenge 50% of the DPPH stable free radical. Although GSH was a slightly better DPPH$^{\bullet}$ scavenger when comparing EC_{50} values, all designed molecules achieved their steady state EC_{50} with a shorter time (T_{EC50}) and thereby showed higher antiradical efficiency than GSH. UPF17 and UPF19, the peptides with α-glutamyl moiety in the backbone, showed the highest antiradical efficiencies, whereas UPF17 was the most active. The T_{EC50} for GSH was 10-fold higher than for UPF17 and the antiradical efficiency of UPF17 was 8.3-fold higher than for GSH. A comparison of T_{EC50} in the pairs of peptides UPF1 and UPF8, UPF17 and UPF25 revealed that amidated peptides (UPF8 and UPF25) need

about two-folds more time to reach a steady state than the similar free acid peptides, T_{EC50} was at 10, 20, 4 and 7 minutes respectively.

4. Toxicity to the cell culture (Paper I)

K562 human erythroleukemia cells were chosen as a basic test to investigate the toxicity of UPF peptides on a cell culture. First, the viability of the K562 cells was determined according to their ATP production after 24 hours of incubation with 200 μM solutions of UPF peptides. 20 μM MTX was used for comparison as a positive control. The ATP production after incubation with peptides compared to untreated cells was as follows: UPF1 98.3 ± 3.1%, UPF6 97.8 ± 0.6%, UPF17 100.7 ± 3%, and UPF19 97.2 ± 5.9%. Hence, UPF peptides did not show any remarkable toxic effects on the viability of K562 cells, whereas only 35% of cells survived MTX treatment.

A second criteria of cell viability was the membrane integrity of K562 cells being assessed by LDH leakage. The effects of the same set of UPF peptides were compared to the effects of MAP. The effects of MAP have been previously studied (Saar and Langel 2006). When the background fluorescence ("no drug") was taken as 0% and the lysis solution Triton X-100-induced signal was taken as 100%, the total cellular LDH leak from K562 cells after MAP treatment was 25%. Meanwhile, none of the UPF peptides caused significant LDH leakage from K562 cells (UPF1 1.3% ± 0.1%, UPF6 1.9 ± 0.1%, UPF17 4.7 ± 0.9%, UPF19 5.1 ± 0.8%). Interestingly, UPF17 and UPF19 disturbed membranes more than peptides with a γ-peptide linkage (UPF1 and UPF6).

5. Effects of UPF peptides on the activity of enzymes (Paper IV)

5.1 Effect of UPF peptides on the activity of GPx and GR

GPx together with GSH are vital for both hydrogen and lipid peroxide elimination, whereas GR reduces formed GSSG back to GSH. The effects of UPF peptides on the activity of GPx were tested by using a purified enzyme or erythrocyte lysate. Changes in the GPx activity after incubation with selected UPF peptides are expressed at each concentration as the percent of basal GPx activity. The results showed that in the range of concentrations used in animal experiments (Kals *et al.* 2008; Põder *et al.* 2004) there is no inhibition of GPx by UPF peptides. For example, the 50 μM UPF1 inhibited GPx activity approximately by 1% and UPF17 3% in the erythrocyte lysate. At substantially higher concentrations, UPF1 and UPF17 decreased the activity of GPx in a concentration dependent manner. Experiments with purified enzyme and erythrocyte lysate showed similar inhibition dynamics. GPx activity after

incubation with 500 μM UPF1 or UPF17 was 71 ± 5% and 51 ± 3% of the basal activity of the purified enzyme. Respective effects on GPx activity by UPF1 and UPF17 in erythrocyte lysates under the same conditions were 76 ± 2% and 61 ± 1% (Paper IV). According to these results, at the same concentrations UPF17 inhibits GPx more than UPF1, and this difference is expressed in the case of both purified enzyme and erythrocyte lysates.

A Lineweaver-Burk plot analysis revealed that the inhibition of GPx by UPF17 at the used concentrations is competitive (Paper IV). The concentration of GSH as the substrate was varied from 0.5–1.5 mM and the concentrations of UPF17 were 0.1 and 0.15 mM.

Neither UPF1 nor UPF17 caused any noticeable change in purified GR enzyme activity at concentrations 0.2–1.0 mM (Paper IV).

5.2 Effect of UPF peptides on the activity of MnSOD

MnSOD is a principal superoxide radical eliminator in the mitochondria, whereas the brain is especially susceptible to OxS. Thus the influence of GSH and UPF peptides on MnSOD activity was investigated on mitochondria isolated from the human temporal cortex. The concentration of added GSH solution was 10 nmol/mg of protein, and incubation times were 5 and 40 minutes. Five minutes of incubation remarkably enhanced MnSOD activity by 21.9 ± 5.8%. After further incubation (40 min), MnSOD activity was decreased back to the same level as the control.

The influence of UPF1 and UPF17 on MnSOD activity was investigated in three different peptide concentrations: 0.5, 1.0 and 5.0 nmol/mg of protein. Incubation times were 5 and 40 minutes. 0.5 nmol/mg of a protein concentration of UPF peptides was the most effective for MnSOD activation. MnSOD activity compared to a basal value (100%) for UPF1 (0.5 nmol/mg of protein) after 5 and 40 minutes were 94.1 ± 0.7% and 116.2 ± 14.7%, and for UPF17 they were 111.5 ± 14.7% and 110.4 ± 10.4%, respectively (Paper IV). These effects were statistically non-significant. At a higher concentration the activity of MnSOD remained unchangeable.

5.3 Effect of UPF peptides on the activity of NAD(P)H oxidase

Neutrophils contain NAD(P)H oxidase, which generates superoxide radical during the oxidative burst. NAD(P)H oxidase inhibitors are investigated because NAD(P)H oxidase over-reactivity is involved in oxidative tissue damages in the case of a high grade inflammatory response. In addition, vascular NAD(P)H oxidase, which has different biochemical characteristics from neutrophil enzyme but shares some common inhibitors, is an important cause of ischemia/reperfusion injury.

Selected UPF peptides were added to the separated PMNs at the concentrations 10 and 100 μM. All peptides tested in paper IV (GSH, UPF1, UPF17) showed a statistically significant inhibitory effect against a PDBu stimulated oxidative burst by a neutrophil NAD(P)H oxidase. A PDBu stimulated signal in the absence of peptides was considered to be 100%. Incubation with 10 μM GSH, UPF1 and UPF17 decreased the signal to 52.9 ± 4.7%, 40.8 ± 4.5% and 50.2 ± 3.8%, respectively. 100 μM peptide concentrations followed analogical differences in the remaining activity (19.3 ± 5.1%, 13.5 ± 2.5%, 18.1 ± 2.9%), indicating that UPF1 shows a tendency to be a slightly more potent NAD(P)H oxidase inhibitor than GSH or UPF17. A change of the γ-glutamyl moiety to the α-glutamyl moiety rather attenuated the inhibitory effect of the UPF peptides (UPF1 compared to UPF17), but it was not statistically significant. Additionally, the influence of 10 μM serine containing UPF6 and UPF19 towards neutrophil NAD(P)H oxidase activity was measured, resulting in the remaining activity 67.9 ± 8.6% and 78.1 ± 9.1%, respectively (Figure 7, data for UPF6 and UPF19 not published). Compared to the methylated tyrosine containing the analogues UPF1 and UPF17, the inhibitory effect of UPF6 and UPF19 was significantly decreased. 100 μM UPF6 and UPF19 showed similar statistically insignificant results compared to other UPF peptides in the NAD(P)H oxidase activity measurements. Only 10 μM UPF19 failed to show statistically significant inhibition compared to PDBu stimulation.

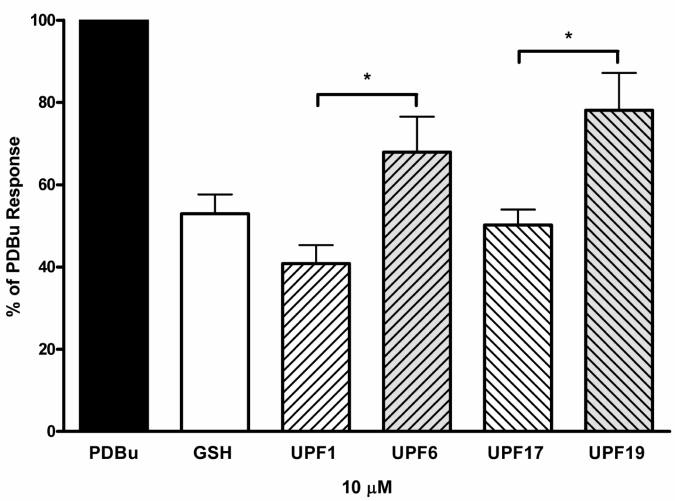

Figure 7. PDBu induced neutrophil NAD(P)H oxidase activity after incubation with GSH, UPF1, UPF6, UPF17, UPF19 on concentration 10 μM. Incubation with PDBu alone corresponds to the maximal superoxide radical production (100%). Values are expressed as mean ± SEM, n=4. Significance of difference compared to controls except for UPF19 ***$P < 0.001$. Significance of difference between UPF1 and UPF6; UPF17 and UPF19 *$P < 0.05$.

DISCUSSION

Glutathione is the main intracellular low molecular weight antioxidant, but it is also important in xenobiotic detoxification, the regulation of the cell cycle, the protection of protein thiol groups and amino acid transport. GSH is represented in the millimolar range in cells and is converted to a disulphide form during RS elimination, whereas GSH/GSSG ratio (mostly 100:1) is the major indicator/ regulator of cellular redox status (Pastore *et al.* 2003). GSH depletion and/or high grade OxS are tightly related to several pathological states, such as neuro-degenerative diseases, CVD, cancer formation, ischemia/reperfusion injuries and high grade inflammatory response. As a consequence, several approaches, such as usage of the precursors of GSH synthesis and the analogues of glutathione, are studied in order to maintain or enhance the intracellular GSH level. UPF peptides are new original glutathione analogues. This study summarizes their design and synthesis and is first of all focused on their primary characterization, which comprises free radical scavenging, stability, a basic toxicity test on a cell culture and the interactions with antioxidative (GPx, GR, and MnSOD) and pro-oxidative enzymes (NAD(P)H oxidase). This information is necessary in order to test in the future the possibility of their maintaining or improving the body defences under the condition of high-grade OxS without impeding the ADS of the human body.

Hydroxyl radical scavenging ability, as the first estimation criteria of antioxidative properties, was measured for the whole UPF peptides library, except for biotin containing UPF7, which had poor water solubility. All UPF peptides were remarkably better hydroxyl radical scavengers than GSH, whereas the modification with the largest impact was the substitution of the γ-glutamyl moiety, characteristic of GSH, to the α-glutamyl moiety. The γ-glutamyl moiety containing UPF peptides had EC_{50} values of hydroxyl radical scavenging between 17–35 μM, whereas the change of the γ-glutamyl moiety to the α-glutamyl moiety drastically decreased EC_{50}. This change improved hydroxyl radical scavenging ability by approximately 500-fold. UPF peptides with the α-glutamyl moiety in their backbone exceeded even the respective property of a widely tested antioxidant melatonin (Pähkla *et al.* 1998). Such a remarkable elevation of antioxidant activity can be explained by the participation of the more available carboxylic acid group in the active state complex, between the peptide and the radical. The addition of different moieties to the N- or C-terminus of glutathione, the exchange of L-amino acids to D-forms, the amidation of peptides or even the change of Cys to Ser did not drastically change the hydroxyl radical scavenging properties of UPF peptides compared to each other. There is no proven explanation as to why the substitution of the thiol group containing Cys, which is supposed to carry a central role in free radical elimination, has no influence on hydroxyl radical scavenging, although it supports a hypothesis about the role of carboxyl group activity.

Based on hydroxyl radical scavenging data and on the supporting literature data (Gozzo *et al.* 1999; Yue *et al.* 1994), a set of UPF peptides were chosen for further investigations. Most often the pair UPF1 and UPF17 was compared, as the methylated tyrosine was considered to be the most promising additional amino acid and the change of the γ-glutamyl moiety against the α-glutamyl moiety was the most effective structural modification comparing the hydroxyl radical elimination results. The DPPH radical elimination ability for the amides of UPF1 and UPF17 (UPF8 and UPF25, respectively) were also measured. Considering the attenuated hydroxyl and the DPPH radical scavenging ability of the amides and the proposed importance of the carboxylic acid group, the amides were discarded for further thesis-related experiments. An additional pair of peptides under broader investigation was the D-serine (one of the very few D-amino acids found in human body) containing UPF6 and UPF19, lacking the aromatic ring in the structure.

Alternatively, hydroxyl radical elimination ability was investigated by CE for a set of UPF peptides that were characterized by half-life or by the rate constants of dimer formation. It was found from these experiments that the rate of the scavenging of the hydroxyl radical in the case of UPF1 was approximately four-fold higher compared to GSH. Surprisingly, UPF17 and UPF19 showed almost equal rate constants with GSH, whereas UPF6 gave an intermediate result. CE results alone suggest that peptides with the γ-glutamyl moiety in the structure should have better antioxidative potential. Additionally, no difference in antioxidativity between the methoxy or the hydroxyl group carrying peptides (UPF1 and UPF15) was observed. Taking both hydroxyl radical elimination results together, when the loss of the hydroxyl radicals was measured (THA method), the α-glutamyl moiety containing UPF peptides showed the highest scavenging ability. Measuring only the concomitant dimerization of UPF peptides (the CE method), peptides with the γ-glutamyl moiety in the structure seemed to be more active. The free radical generation in the CE takes place under similar physiological-like conditions to the THA method, and the reaction is quickly stopped (within five seconds) when the mixture is introduced into the capillary.

The DPPH radical scavenging activity of UPF1, UPF6, UPF8, UPF17, UPF19 and UPF25 in the cell free system was determined and their activity compared to GSH. Based on the times required for a half reaction (T_{EC50}), as determined by Sanchez-Moreno and co-workers, the kinetic behaviour of the antioxidant compound was classified as follows: < 5 min (rapid), 5–30 min (intermediate), and > 30 min (slow) (Sanchez-Moreno *et al.* 1998). According to this classification, UPF17 was rapid; UPF1, UPF6, UPF8, UPF19 and UPF25 were intermediate; and GSH was a slow antioxidant. T_{EC50} for UPF1 was 4-fold and for UPF6 2-fold shorter compared to GSH. T_{EC50} for UPF17 and UPF19 were 2.5-fold shorter than for UPF1 and UPF6, respectively. The DPPH radical was scavenged by GSH and UPF peptides through the donation of hydrogen to form the stable DPPH-H. Similarly to the THA method, DPPH radical elimination experiments also confirmed the higher activity of the α-glutamyl

moiety containing UPF peptides. The results of free radical scavenging experiments suggest that more mechanisms than only electron donation by the thiol group may be involved in the free radical elimination process. The expected electron donation by UPF peptides converts free radicals to more stable products and terminates a radical chain reaction, but the complex mechanism of free radical elimination must be explained.

The stability of UPF peptides was observed to determine their status in the experiments carried out and to predict their status at storage and *in vivo* environment. It is a well-known fact that glutathione and peptides with the free thiol group easily oxidize in the solution. The electronic structure of the sulphur atom elicits the high reactivity of the thiol group towards nucleophilic addition, redox reactions and metal chelation. A key issue in the stability of UPF peptides also turned out to be the formation of disulphide bridges resulting in the dimeric form of the peptide. Dimer formation was followed for selected UPF peptides and compared to GSH in different environments, such as water, physiological solution, a phosphate buffer, a solution of $CuSO_4$ and a solution of H_2O_2. Dimerization was a slow (taking days) process, although it occurred faster in the presence of salt. UPF peptides with the γ-glutamy moiety (UPF1) dimerized faster than a respective analogue with the α-glutamyl moiety (UPF17). The first signs of dimerization in the phosphate buffer occurred after four hours. These results show that dimerization can not influence the results presented in the current work, as the solutions were made *ex tempore*. Circular dichroism experiments showed that UPF peptides do not have any secondary structure in the water solution and do not aggregate even at a 1 mM concentration (Paper II). However, it must be considered that the dimerization process is disturbingly fast if the solution is administered by infusion over several days. Based on current *in vitro* stability investigations in the presence of salts, a remarkable quantity of UPF peptides may be in disulphide form *in vivo* as well. In addition, the results showed that the mixed dimer formation of UPF peptides with GSH may be favoured. Despite this, the protective potency of UPF1 in different animal models is proven (Kals *et al.* 2008; Põder *et al.* 2004). These results together indicate that the quantity of the monomeric form is sufficient to fulfill its protective action; or alternatively, the dimeric form of UPF peptides may also carry bioactivity.

GSH and UPF peptides (UPF1, UPF6, UPF15, UPF17, UPF19) showed a similar speed of oxidation in the presence of H_2O_2. At low concentrations H_2O_2 is rather inert, but in current experiments the concentration was sufficient for oxidizing the peptides. Similar half-lives suggest that UPF peptides should *in vivo* react directly with H_2O_2 to about the same extent as GSH (which is modest), indicating that H_2O_2 elimination is not their predominate mechanism to express antioxidative properties. Differences occurred in the presence of $CuSO_4$, where UPF1 and UPF15 showed about two-fold faster oxidation than GSH. All the peptides were more stable in the presence of $CuSO_4$ than in the H_2O_2 milieu, and this difference was expressed more for GSH than for UPF peptides. The supposed ability of UPF peptides to chelate metal ions more

effectively than GSH may help to force back the pro-oxidative activity of transition metal ions *in vivo*. It may be speculated as to how much the Cu^{2+} chelation ability of UPF peptides may influence the scavenging of hydroxyl radicals generated by the reaction between Cu^{2+} and hydrogen peroxide. Still, it would not attenuate the importance of the gathered information, as transition metal ions are predominant mediators of hydroxyl radical formation in the human body. Considering the potential participation of copper chelation, the term hydroxyl radical elimination would be more correct in order to characterize obtained results than scavenging ability, as "elimination" includes the suppression of hydroxyl radical formation as well. Nevertheless, the direct scavenging should give the dominant part of the hydroxyl radical elimination effect, because free radical generation is supposed to be a much faster process than metal chelation by thiols.

The non-toxic influence of UPF1 was previously confirmed on primary cerebellar granule cells (Põder *et al.* 2004). Primary toxicity scan of selected UPF peptides was performed in the current work. UPF peptides did not show any influence on the viability of K562 cells, and practically did not disturb the plasma membrane structure of the cells. For a disturbance of membranes, UPF peptides are too short; but again, the peptides which included α-peptide bond in the structure had slightly more effect. At the same time, the addition of more hydrophobic moiety into the sequence did not influence the membrane perturbance.

Applicable synthetic antioxidants should not interfere with the functionality of body ADS. Accordingly, one of the aims of this work was to study the effects of UPF peptides on the natural enzymatic defence against OxS. Higher, non-pharmacological concentrations of UPF peptides were used to better investigate the structure-related effects. At low concentrations (up to 50 μM), similar to what are used in *in vivo* experiments, UPF peptides did not inhibit GPx activity and neither UPF1 nor UPF17 influenced the mRNA expression of human blood monocytes. At higher concentrations, both UPF peptides inhibited GPx activity concentration-dependently, whereas the α-peptide bond containing UPF17 had the strongest inhibitory effect. *In silica* modeling is further needed to study spatial rearrangements of the enzyme. In the case of a purified enzyme, the inhibitory effects of UPF peptides were more expressed than in the erythrocyte lysate, and this tendency is more pronounced at lower concentrations. This could be explained by the additional number of interfering components in the erythrocyte lysate reducing the acting concentration of the peptide. As the measurement of GPx activity comprises two reactions – creating GSSG by GPx and the further conversion of GSSG by GR, which depletes NADPH and leads to signal decrease – the exact cause of the signal decrease due to UPF peptides must be further investigated. The results shown on the Lineweaver-Burk plot can be caused by the competitive reaction between GSH and UPF17 in the active centre of GPx.

UPF peptides showed no influence on GR activity, which means they do not disturb the conversion of GSSG back to GSH. It was also made possible to

measure GPx activity with the indirect method described above. Still, there is the question as to whether the dimeric form of UPF peptides or their heterodimer with GSH could be a substrate for GR. The relevance of this information is the following – can the UPF peptides be recycled back to the monomeric form, which supposedly carries the bioactivity? Stability investigations indicate that the spontaneous dimerization of UPF peptides is too slow a process to influence the results of GPx and GR measurements.

MnSOD is the immediate eliminator of superoxide radicals produced constantly by the mitochondrial respiratory chain. Testing the influence of UPF peptides on MnSOD activity is critical, as any suppression of MnSOD activity leads to excessive quantities of RS that may impair the mitochondrial function, leading to reduced energy production, etc. UPF1 and UPF17 showed a tendency of MnSOD activation, although it was not statistically significant. It seems that the γ-glutamyl moiety containing UPF1 needed more time for MnSOD activation, whereas the α-glutamyl moiety containing UPF17 exerted its influence already in the 5th minute. Only the lowest used concentration (0.5 nmol/mg of protein) showed a certain activating effect; at higher concentrations, peptides did not have any influence on MnSOD activity. A study performed by Matsuda *et al.* indicated that human liver MnSOD is composed of four identical subunits, in which one cysteine (Cys196) is readily reactive towards thiols, whereas the other (Cys140) is hidden inside the molecule (Matsuda *et al.* 1990). In light of these data, some predictions about the UPF peptides and brain MnSOD interaction mechanisms can be given. Expecting that the thiol groups of MnSOD have different activities toward UPF peptides, UPF at a higher concentration (more than 0.5 nmol/mg of protein) may react with additional thiol groups. A concomitant change in MnSOD conformation leads to decreased activity. The incubation of the mitochondrial fraction with GSH gave a statistically significant increase in MnSOD activity after five minutes. After 40 minutes, the MnSOD activity was equal to basal again. Speculatively, this could be explained by the leaving of the GSH from the active site after a longer time of incubation. An alternative explanation as to the loss of activity in the 40 minutes incubation with GSH may also be that this time is sufficient for reacting with additional thiol groups.

In general, the moderate stimulation of antioxidant enzymes should be considered as a positive effect for a potential antioxidative protector-molecule. At the same time, it must be considered that the human body antioxidative defence is a cooperation of different enzymes. Superoxide dismutases catalyse the dismutation of the superoxide radical into hydrogen peroxide, but if hydrogen peroxide production overcomes the ability of GPx or the catalase to eliminate its excess, it could lead to OxS. The hypothesis that increasing the concentration of SOD alone might enhance OxS resistance is disconfirmed by several studies (Amstad *et al.* 1994; Giorgio *et al.* 2007). In light of these results, it is advantageous that UPF peptides had no statistically significant stimulating effect on MnSOD activity in the isolated mitochondria of the human brain temporal cortex. In addition, it has been shown that UPF peptides do not

alter the expression level of MnSOD in the human blood monocytes (Paper IV). However, increased MnSOD gene expression induced by small molecular weight thiols has been shown before in lung tissue by NAC (Nagata *et al.* 2007).

NAD(P)H oxidases are the main sources of superoxide radicals in neutrophils and vascular tissues, and although having a physiological defensive or regulatory role respectively, their over-reactivity is related to pathological states as high grade inflammatory response, ischemia/reperfusion injuries and/or changes in vascular tone. S-thiolation is accompanied by a stimulation of respiratory burst and is correlated with respiratory burst intensity (Seres *et al.* 1996). Many NAD(P)H oxidase inhibitors mediate their influence through conjugation with thiol groups essential for NAD(P)H oxidase activation; for review see Ref. (Cross 1990). This has led to the thought that NAD(P)H oxidase inhibition by UPF peptides could also be mediated by binding the thiol groups vicinal for enzyme activation. Controversial research has shown that the binding of thiol groups does not always lead to an inhibition of NAD(P)H oxidase. Moriguchi *et al.* induced S-thiolation by pretreating the neutrophils with a direct thiol oxidizing compound diamide and showed the enhanced release of superoxide anion by NAD(P)H oxidase after binding the thiol groups (Moriguchi *et al.* 1996). The different effects of thiol binding could be explained by the exact location of the modification and the previous redox status of the enzyme. It is generally recognized that thiol oxidants inhibit phagocytic NAD(P)H oxidase, but regarding a vascular enzyme, it depends on the used thiol and NADPH- or NADH-driven signal. For example, a thiol group containing the glutathione precursor NAC has been shown to inhibit vascular NAD(P)H oxidase when used alone, but has some recovery effect on enzyme activity when used together with other NAD(P)H oxidase inhibitors (Janiszewski *et al.* 2000). The results obtained with UPF peptides from experiments with neutrophil NAD(P)H oxidase can not be automatically expanded to the vascular enzyme, although these isoenzymes share several common inhibitors. It is widely accepted that NAD(P)H oxidase inhibitor apocynin inhibits NAD(P)H oxidase in phagocytic cells but it has been proposed that apocynin increases ROS generation in vascular tissues, because it requires conversion into an active form (Vejrazka *et al.* 2005). Comparing the structural similarities of UPF peptides with low molecular weight NAD(P)H oxidase inhibitors, UPF1 and apocynin both contain a methoxy moiety in the aromatic structure. It is not known whether the UPF peptides are able to penetrate through the neutrophil membrane; hence at the moment a more exact location of binding can not be predicted. The thiolation of free thiol groups by UPF peptides can still be questioned as a potential inhibition mechanism. The inhibition of NAD(P)H oxidase activity was attenuated when methylated tyrosine was replaced by serine and was less dependent on the α-/γ-glutamyl moiety exchange, although the γ-peptide bond seems to be slightly more preferred. It may be assumed that the hydrophobic nature of methylated tyrosine facilitates the interaction with neutrophil membranes. Previously it has been excluded that UPF peptides could

be potential non-specific superoxide radical scavengers (data not published). Generating superoxide via xanthine/xanthine oxidase reaction, UPF peptides failed to significantly decrease the superoxide radical production. This confirms that the signal decrease after the incubation of PMNs with UPF peptides is caused by an interaction with NAD(P)H oxidase. The results of enzyme activity investigations suggest that the α-/γ-glutamyl moiety exchange gives no considerable difference in influencing GR, MnSOD and NAD(P)H oxidase activities. Statistical difference was found only in the case of GPx inhibition in the favor of the α-glutamyl moiety containing peptide.

In conclusion, the library of novel nontoxic antioxidants has been designed and synthesized, which showed very potent antioxidativity compared to GSH and do not significantly influence on principle antioxidative enzyme activities. Moreover, UPF peptides suppress the activity of potentially pro-oxidative NAD(P)H oxidase. The results obtained from the current research confirm that investigated UPF peptides can be used by themselves or as a lead towards the design of protector-molecules that may have an impact concerning the correction of profound high grade OxS-related conditions.

CONCLUSIONS

1. A library of novel glutathione (GSH) analogues (arbitrarily called UPF peptides) consisting of 17 compounds was designed and synthesized. To test structure-functional relationships, different approaches – such as linking a fourth amino acid to a GSH backbone, replacing the native γ-glutamyl moiety in the GSH backbone with α-glutamyl moiety, using D-amino acids instead of L-isomers and the amidation of the terminal carboxyl group – were applied. *O*-methyl-L-tyrosine was mostly used as the fourth amino acid as various good low molecular weight antioxidants also include methoxy moiety attached to aromatic ring in their structure.
2. All UPF peptides had a substantially effective hydroxyl radical scavenging ability compared to GSH. The substitution of the γ-glutamyl moiety to α-glutamyl moiety drastically improved the hydroxyl radical scavenging properties of UPF peptides, but other structural modifications had limited effects. Measuring only the concomitant dimer formation of UPF peptides, γ-glutamyl moiety containing UPF peptides (UPF1, UPF6) had a higher antioxidative potential than GSH and peptides including α-glutamyl moiety (UPF17, UPF19). These controversial results show that more mechanisms, in addition to hydroxyl radical elimination by the thiol group, may be involved.

   GSH had a slightly lower EC_{50} of DPPH radical scavenging than UPF peptides, but regarding antiradical efficiencies (reflecting the speed of DPPH radical scavenging), UPF peptides exceeded GSH by antioxidative potential, whereas UPF17 was the most effective one.
3. UPF peptides were relatively stable as remarkable amount of dimeric forms of these molecules were not detected before 48 hours in physiological solution and at least half of the peptide was in monomeric form after a week. However, the UPF peptides dimerized faster than GSH in water, physiological solution, $CuSO_4$ solution and phosphate buffer solution. The dimerization of all peptides, including GSH, was favoured by the salts and was most expressed in the case of $CuSO_4$. γ-Glutamyl moiety containing UPF1 had a higher dimerization rate than UPF17 with α-glutamyl moiety. In the presence of hydrogen peroxide UPF peptides (UPF1, UPF6, UPF15, UPF17, and UPF19) and GSH showed a similar dimerization speed which was faster compared to that in the saline solutions. The higher dimerization rate of UPF peptides suggests an elevated reactivity of the thiol group and, through it, higher antioxidative potential.

   UPF peptides (UPF1, UPF6, UPF17, and UPF19) did not suppress the viability of K562 human erythroleucemia cells.
4. Selected UPF peptides had following effects on antioxidative enzymes – UPF peptides did not affect the activity of GR, showed a slight tendency to increase MnSOD activity and inhibited GPx activity only at high concentrations and this effect was more expressed for α-glutamyl moiety containing UPF17. In addition, UPF peptides inhibited the potentially pro-oxidative human neutrophil NAD(P)H oxidase.

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SUMMARY IN ESTONIAN

Antioksüdantsed glutatiooni analoogid (UPF peptiidid) – molekulaarne disain, struktuur-aktiivsuse seosed ja protektiivsete omaduste testimine

Glutatioonil (GSH) on inimorganismis rida ülesandeid (ensüümvalkude aktiivsuse regulatsioon, ksenobiootikumide detoksifikatsioon, hemoglobiini kaitse denaturatsiooni eest, valkude tioolrühmade taastamine, rakkude signalisatsioon, jne.). Arvestades lisaks ka asjaolu, et GSH on keskne rakusisene antioksüdant ning sügaval kestval (*high grade*) oksüdatiivsel stressil on oluline roll haiguste/kahjustuste (südame-veresoonkonna haigused, neurodegeneratiivsed haigused, vähkkasvajad, isheemia/reperfusioonikahjustused jt.) patogeneesis, püütakse leida GSH süsteemi tööd toetavaid strateegiaid.

Käesolevas töös keskenduti esmalt uute GSH analoogide disainile, mille tulemusena sünteesiti seitsmeteistkümne ühendiline "raamatukogu" (kokkuleppelise nimetusega UPF peptiidid). Struktuuri ja funktsioonide seoste uurimiseks kasutati järgnevaid modifikatsioone: tripeptiidsele GSH molekulile neljanda aminohappe lisamine, γ-glutamüüljäägi asendamine α-glutamüüljäägiga, L-aminohapete asendamine D-aminohapetega ja/või C-terminaalse karboksüülrühma amideerimine. Kuna GSH analoogid peaksid praktikas kasutatavates hulkades olema mittetoksilised, stabiilsed ja võimalusel paremate antioksüdantsete omadustega kui GSH, uuriti käesolevas töös UPF peptiidide vastavaid omadusi.

Kõik UPF peptiidid olid efektiivsemad hüdroksüülradikaali (reaktiivseim vaba radikaal inimorganismis) elimineerijad kui GSH. γ-Glutamüüljääki sisaldavad UPF peptiidid olid umbes 60-korda paremad hüdroksüülradikaali elimineerijad kui GSH ning γ-glutamüüljäägi asendamine α-glutamüüljäägiga parandas UPF peptiidide efektiivsust veel umbes 500-korda. Järgnevatesse uuringutesse valiti komplekt UPF peptiide lähtuvalt hüdroksüülradikaali püüdmisvõimest ja mõningates teistes uuringutes leitud omadustest (näiteks lahustuvus). Kuna glutatioonist moodustub nii reaktiivsete osakeste elimineerimisel kui ka säilitamisel dimeerne vorm (GSSG), uuriti ka UPF peptiidide dimerisatsiooni. Määrates hüdroksüülradikaali püüdmisvõimet tekkiva dimeerse vormi kvantifitseerimise kaudu selgus, et hoopis γ-glutamüüljääki sisaldavad peptiidid (UPF1, UPF6) omasid kõrgemat antioksüdatiivset potentsiaali, kui vastavad α-glutamüüljäägiga analoogid (UPF17 ja UPF19). Need vastuolulised tulemused viitavad võimalusele, et hüdroksüülradikaali püüdmine toimub lisaks tioolrühma osalusele ka veel teiste mehhanismide kaudu. Mitmekülgse info saamiseks UPF peptiidide vabu radikaale elimineerivate omaduste kohta määrati ka nende ühendite DPPH radikaali püüdmisvõime võrrelduna glutatiooniga. UPF peptiidide DPPH radikaali eliminatsiooni EC_{50} väärtused ei omanud sisulist erinevust GSH vastavate näitajatega. Samas UPF peptiidid ületasid

glutatiooni DPPH radikaali eliminatsiooni kiiruses, teisisõnu nende antiradikaalne efektiivsus oli oluliselt suurem.

Nagu ka glutatioonil oli UPF peptiidide lahuste stabiilsuse seisukohalt kõige kriitilisem dimerisatsioon tioolrühmade kaudu. GSH ja UPF peptiidid säilusid valdavas osas monomeerse vormina füsioloogilises lahuses vähemalt 48 tundi ning ka nädala möödudes oli monomeeri osakaal vähemalt 50%. UPF peptiidid dimeriseerusid kiiremini kui GSH ning mõlema ühendi puhul oli see protsess soodustatud soolade juuresolekul (NaCl, $CuSO_4$ või fosfaatpuhver). Sealjuures UPF1 (Tyr(Me)-γ-Glu-Cys-Gly) dimeriseerus vesilahuses ja füsioloogilises lahuses kiiremini kui α-glutamüüljääki sisaldav UPF17 (Tyr(Me)-α-Glu-Cys-Gly). UPF peptiidide (UPF1, UPF6, UPF15, UPF17, UPF19) ja GSH dimerisatsiooni kiirus vesinikperoksiidi juuresolekul ei erinenud, kuid väljendus tugevamalt võrreldes dimerisatsiooniga soolalahustes. UPF peptiidide kiirem dimerisatsioon viitab tioolrühma suuremale aktiivsusele ja seeläbi parematele antioksüdantsetele omadustele.

Toksilisuse mudelina kasutati inimese K562 erütroleukeemia rakke. UPF peptiidid ei pärssinud K562 rakkude eluvõimelisust nendes kontsentratsioonides, mida kasutati käesoleva töö teistes eksperimentides.

Perspektiivsed on need protektormolekulid mis ei häiri inimorganismi antioksüdantsete kaitsemehhanismide tööd. Seetõttu testiti väljavalitud UPF peptiidide toimet inimorganismi antioksüdantsetele võtme-ensüümidele: glutatiooni peroksüdaasile (GPx), glutatiooni reduktaasile (GR) ja mangaan-superoksiidi dismutaasile (MnSOD). UPF peptiidid ei muutnud GR aktiivsust ja inhibeerisid GPx aktiivsust oluliselt ainult kõrgematel kontsentratsioonidel, mida polnud vaja kasutada varasemates loomkatsetes UPF1-ga. Inhibeeriv efekt oli rohkem iseloomulik α-glutamüüljääki sisaldavale UPF17-le. Disainitud ühendid (UPF1, UPF17) ei mõjutanud MnSOD aktiivsust. Viimane informatsioon on eriti oluline kuna mitokondriaalne hingamisahel on superoksiidi radikaali põhikoguse tootja ja MnSOD on tekkinud radikaali ainus otsene elimineerija mitokondrites.

NAD(P)H oksüdaas produtseerib superoksiidi radikaali fagotsütaarsetes rakkudes ja on oluline organismi kaitsevõime seisukohalt. Liigne NAD(P)H oksüdaasi aktivatsioon põhjustab aga koekahjustusi, seepärast on NAD(P)H oksüdaasi aktiivsuse pärssimine teatud olukordades (anormaalne põletikureaktsioon, isheemia/reperfusioonikahjustused) hädavajalik. UPF peptiidid inhibeerisid NAD(P)H oksüdaasi aktiivsust, sealjuures vähendas metüleeritud türosiini asendamine (UPF1, UPF17) seriiniga (UPF6, UPF19) ühendite võimet inhibeerida NAD(P)H oksüdaasi.

Seega disainiti ja sünteesiti uute mittetoksiliste antioksüdantide raamatukogu (UPF peptiidid), mille esindajate antioksüdatiivne efektiivsus ületab oluliselt glutatiooni efektiivsuse. Uuritud ühendid ei pärssinud inimorganismi antioksüdantsete võtme-ensüümide aktiivsust, inhibeerisid aga potentsiaalselt prooksüdatiivse NAD(P)H oksüdaasi aktiivsust. Käesoleva töö tulemused näitavad, et UPF peptiidid on unikaalsed perspektiivsed protektor-peptiidid, mis võivad leida rakendust kestva sügava oksüdatiivse stressi korrektsioonis.

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PUBLICATIONS

CURRICULUM VITAE

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Education

2005–2009	PhD studies, Department of Biochemistry, Faculty of Medicine, University of Tartu, Estonia
2007	Master's program in biomedicine (MSc), Faculty of Medicine, University of Tartu, Estonia
1997–2002	Pharmacist's study, Faculty of Medicine, University of Tartu, Estonia
1994–1997	Tartu Karlova Gymnasium

Professional employment

2008–	Extraordinary Research Fellow, Department of Biochemistry, Faculty of Medicine, University of Tartu, Estonia
2005–	OÜ TePiBa
2003–2008	Specialist, Department of Biochemistry, Faculty of Medicine, University of Tartu, Estonia
2002–2004	Pharmacist, Akos Apteek OÜ, Tartu, Estonia

Scientific work

Main field of investigation: Design and synthesis of novel antioxidative glutathione analogues and investigation of the protective properties in high grade oxidative stress related events; limitation of oxidative stress caused by NAD(P)H oxidase. 4 scientific articles in peer-reviewed journals, 5 posters and 1 oral presentation.

Awards

2007 Estonian National Contest for Young Scientists at University Level. First Prize in Medicine at the level of MSc/MA thesis
2007 The scholarship of Friedrich Carl von Krueger

Teaching experience

2004–2009 218 hours of auditorial work, seminars/practical works
Two research work of pharmacy studies supervised (equal to master degree) on 2006 and 2008 year. University of Tartu, Faculty of Medicine, Department of Pharmacy.

Special courses

2004 Stereology course, Tartu, Estonia
2004 Meeting of peptide-research groups, Stockholm University, Sweden
2006 Mass-spectrometry course, Tartu, Estonia
2007 Äkta Purifier HPLC course, Tartu, Estonia
2007 Proteomics/MS course, Tallinn/Tartu, Estonia
2009 FELASA C-category Competence Course of Laboratory Animal Science, Tartu, Estonia

Membership of professional societies

2005– International Union of Biochemistry – as a member of the Estonian Biochemical Society.
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1. Kersti Ehrlich, Katrin Ida, Riina Mahlapuu, Ceslava Kairane, Ingrid Oit, Mihkel Zilmer, Ursel Soomets. Characterization of UPF peptides, members of the glutathione analogues library, on the basis of their effects on oxidative stress related enzymes. Accepted by Free Radical Research on 20 March 2009.
2. Kersti Ehrlich, Säde Viirlaid, Riina Mahlapuu, Külliki Saar, Tiiu Kullisaar, Mihkel Zilmer, Ülo Langel, Ursel Soomets. Design, synthesis and properties of novel powerful antioxidants, glutathione analogues. Free Radical Research 2007;41(7):779–787.

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9. Ehrlich, Kersti; Mahlapuu, Riina; Zilmer, Mihkel; Soomets, Ursel (2006). Synthetic glutathione analogues as potential eliminators of superoxide radical, produced by neutrophil NAD(P)H oxydase. Eesti Arst, 9, 616 (in Estonian). In addition, oral presentation on Annual Conference of Faculty of Medicine, University of Tartu, 2006.
10. Ehrlich, K.; Mahlapuu, R.; Viirlaid, S.; Zilmer, M.; Langel, Ü.; Soomets, U. (2005). Antioxidative properties of novel antioxidant UPF1 and its analogues. 1st BBBB Conference on Pharmaceutical Sciences, Siofok, Hungary, September 26–28., 2005, 132–133.

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1997–2002	Tartu Ülikooli arstiteaduskond, proviisoriõppe põhiõpe
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Teenistuskäik

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Teadustegevus

Peamine uurimisvaldkond: Uute antioksüdantsete glutatiooni analoogide disain ja süntees ning protektiivsete omaduste uurimine liigse oksüdatiivse stressi seisundites; NAD(P)H oksüdaasi poolt põhjustatud oksüdatiivse stressi limiteerimine.

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2004–2009 218 tundi auditoorset tööd seminarid/praktikumid.
Kaks proviisoriõppe lõputööde juhendamist (magistrikraadiga võrdsustatud kvalifikatsioon), kaitstud aastatel 2006 ja 2008. Tartu Ülikooli arstiteaduskond, farmaatsia instituut.

Erialane enesetäiendus

2004 Stereoloogia kursus, Tartu, Eesti
2004 Peptiidigruppide kokkusaamine, Stockholmi Ülikool, Rootsi
2006 Mass-spektromeetria kursus, Tartu, Eesti
2007 Äkta Purifier HPLC koolitus, Tartu, Eesti
2007 Proteoomika/MS kursus, Tallinn/Tartu, Eesti
2009 Katseloomateaduse FELASA C-kategooria kursus, Tartu, Eesti

Erialased organisatsioonid

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1998–2003 Tartu Ülikooli Rohuteaduse Seltsi liige

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Teaduslikud artiklid eelretsenseeritavates ajakirjades

1. Kersti Ehrlich, Katrin Ida, Riina Mahlapuu, Ceslava Kairane, Ingrid Oit, Mihkel Zilmer, Ursel Soomets. Characterization of UPF peptides, members of the glutathione analogues library, on the basis of their effects on oxidative stress related enzymes. Accepted by Free Radical Research on 20 March 2009.
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Konverentsi teesid

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10. Ehrlich, K.; Mahlapuu, R.; Viirlaid, S.; Zilmer, M.; Langel, Ü.; Soomets, U. (2005). Antioxidative properties of novel antioxidant UPF1 and its analogues. 1st BBBB Conference on Pharmaceutical Sciences, Siofok, Hungary, September 26–28., 2005, 132–133.

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