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Design, synthesis and properties of novel powerful antioxidants, glutathione analogues

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Abstract
Glutathione (GSH) is the major low-molecular weight antioxidant in mammalian cells. Thus, its analogues carrying similar and/or additional positive properties might have clinical perspectives. Here, we report the design and synthesis of a library of tetrapeptidic GSH analogues called UPF peptides. Compared to cellular GSH our designed peptidic analogues showed remarkably higher hydroxyl radical scavenging ability (EC_{50} of GSH: 1231.0 ± 311.8 μM; EC_{50} of UPF peptides: from 0.03 to 35 μM) and improved antiradical efficiency towards a stable α,α-diphenyl-β-picrylhydrazyl (DPPH) radical. The best of UPF peptides was 370-fold effective hydroxyl radical scavengers than melatonin (EC_{50}: 11.4 ± 1.0 μM). We also found that UPF peptides do not influence the viability and membrane integrity of K562 human erythroleukemia cells even at 200 μM concentration. Dimerization of GSH and UPF peptides was compared in water and in 0.9% saline solutions. The results, together with an earlier finding that UPF1 showed protective effects in global cerebral ischemia model in rats, suggest that UPF peptides might serve both as potent antioxidants as well as leads for design of powerful non-peptidic antioxidants that correct oxidative stress-driven events.

Keywords: *Oxidative stress, glutathione, free radicals, hydroxyl radical, DPPH*

Abbreviations: *DCC, dicyclohexylcarbodiimide; DCM, dichloromethane; DIEA,* N,N-*diisopropylethylamine; DMF,* N,N-*dimethylformamide; DMSO, dimethylsulphoxide; DPPH˙, α,α-diphenyl-β-picrylhydrazyl; EDT, 1,2-ethanedithiol; EMS, ethylmethylsulfide; Fmoc, 9-fluorenylmethoxycarbonyl; GGT, γ-glutamyltransferase; GSH, reduced (monomeric) form of glutathione; GSSG, oxidized (dimeric) form glutathione; GST, glutathione S-transferase; HF, hydrofluoric acid; HKR, HEPES-buffered Krebs-Ringer solution; HOBt, 1-hydroxybenzotriazole; LDH, lactate dehydrogenase; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; MAP, model amphipathic peptide; MBHA, 4-methylbenzhydrylamine; MTX, methotrexate; TBTU, 2-(1H-benzsotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; tert-Boc, tert-butyloxycarbonyl; TFA, trifluoroacetic acid; THA, terephthalic acid; TIS, triisopropylsilane; UPFs, UPF peptides*

Introduction

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

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features: a γ-glutamyl linkage and a sulphydryl group. GSH as a nucleophile reacts with endogenous and exogenous electrophile compounds, a majority of these detoxification reactions are mediated by glutathione S-transferases (GST) [1]. In addition GSH helps to maintain the sulphydryl groups of many proteins in the functional, reduced form. GSH oxidizes to dimeric form (GSSG) via reacting non-enzymatically with certain reactive species (hydroxyl radical, hypochlorous acid and peroxynitrite) or during the elimination of peroxides being a co-factor in glutathione peroxidase [2,3]. GSH depletion changes the GSH redox status in the cell, which is defined as the GSH/GSSG ratio (at normal conditions 100 or more) [4,5]. This redox ratio is involved in the regulation of most cellular metabolic processes and in the activation of the redox-sensitive transcriptional elements [6].

Both GSH depletion and the high-grade oxidative stress occur in a wide variety of conditions. It includes several chronic diseases (cardiovascular diseases, neurodegenerative diseases, cancer formation and HIV) and acute clinical conditions (inflammation, infarction, stroke, organ transplantation, ischemia/reperfusion injury, renal failure, lung injury and complications of surgical operations). Stress, aging and strenuous physical exercises have also been considered [7–11]. Participation of high grade oxidative stress in many pathological events creates a requirement for new molecules with improved antioxidant activities.

Due to the versatile role of GSH, different strategies have been applied to maintain the functionality of the GSH system. One of the research objectives is how to restore the intracellular GSH level that may be potentially useful in different clinical conditions named above. Reaching the sufficient cellular GSH concentration (up to some millimolar) by administering GSH itself is complicated, because of its rapid degradation in the digestive system and the difficulties with direct uptake into different cell types. Some positive results with administering of exogenous GSH have been shown, like the preservation of the renal function after ischemic renal injury [12]. 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 has toxicity problems [13], the cysteine precursors, for example, *N*-acetyl-L-cysteine have been used [14,15]. Due to versatile roles of GSH, the great number of GSH analogues with extremely different properties have been synthesized [16].

Various modifications of GSH molecule have been performed to improve its stability and cellular uptake. A 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 [17]. Substitution of the amino group at the GSH molecule N-terminus with pyrrole ring gives new antioxidants that due to steric hindrance do not inhibit the GSH reductase nor the glutathione peroxidase [18]. Replacing the native γ-glutamyl moiety with the *cis*- or *trans*-4-carboxyl-L-proline residue gives conformationally-rigid skeleton and makes this GSH analogue resistant to γ-glutamyltransferase (GGT) degradation [19]. The outstanding group of GSH analogues are cysteine-substituted S-nitrosoglutathiones that have been investigated based on physiological roles of both GSH and nitric oxide, for review see Ref. [20].

In some clinical situations like cancer therapies, diminishing the GSH level is the goal. Overexpression 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 [21]. In this way, a large number of GSH analogues have been designed to inhibit different GST isoenzymes: the phosphono analogues [22] and the peptidometic analogues that are stable towards GGT, the main enzyme of GSH breakdown [23]. One of the latest and more successful GSH analogues in cancer therapy TLK 286, is in clinical trials [24]. Some designed GSH analogues act as glyoxalase inhibitors and have shown potent antiproliferative and anti-tumour activity [25]. Still, the improvement of GSH analogues stability towards peptidases and proteases stands as general problem. One possible solution to overcome this problem is through the cyclization of GSH molecule. Such analogues have been tested for antitumor activity [26].

Previously, we have designed UPF1 peptide by adding the non-coded amino acid *O*-methyl-L-tyrosine (Tyr(Me)) to the N-terminus of GSH

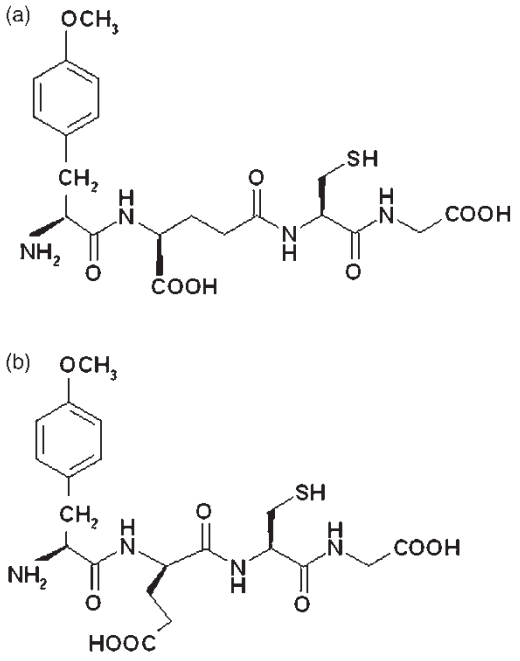

Figure 1. General structure of UPF1 (a) and UPF17 (b).

(Figure 1(a)). We have shown that UPF1 is 60-fold better hydroxyl radical scavenger than GSH [27] and may also act as a modulator of G proteins in frontocortical membrane [28]. UPF1 has been already investigated in different *in vivo* experimental models in rats: global brain ischemia [27], Langendorff model with isolated heart [29], 5/6 nephrectomia model (unpublished data), and it has been shown to have protective effect. On the basis of gathered information the aim of the present study was to design a small library of more powerful antioxidants than GSH, find structural features of compounds that are responsible for increase in antioxidativity and investigate stability and toxicity of the UPF peptides, to find the most promising analogues for the *in vivo* experiments. By determining the reactivity of UPF peptides *in vitro*, this study is the first stage to create presumption for following functional studies using animal models.

Materials and methods

Reagents and solvents

9-Fluorenylmethoxycarbonyl (Fmoc)–Gly–Wang resin, Rink Amide MBHA (*p*-methylbenzhydrylamine) resin, Fmoc-protected amino acids, Boc-protected amino acids, dicyclohexylcarbodiimide (DCC), 1-hydroxybenzotriazole (HOBt), 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) were purchased from Novabiochem, Switzerland; *N*,*N*-dimethylformamide (DMF), dichloromethane (DCM), dimethylsulphoxide (DMSO), *N*,*N*-diisopropylethylamine (DIEA), acetonitrile from BDH Laboratory Supplies, England; trifluoroacetic acid (TFA), ethylmethylsulfide (EMS), triisopropylsilane (TIS) from Fluka; hydrofluoric acid (HF) from AGA, Sweden; 1,2-ethanedithiol (EDT), terephthalic acid (THA), $CuSO_4 \cdot 5H_2O$, disodiumphosphate, hydrogen peroxide 30% (w/w, water solution), α,α-diphenyl-β-picrylhydrazyl (DPPH radical) from Sigma-Aldrich, Germany. Methotrexate (MTX) was from Amersham Biosciences AB (Uppsala, Sweden), model amphipathic peptide (MAP) was from AC Scientific Inc. (Duluth, GA, USA). DMF and DCM were stored on molecular sieves (∅ 4 Å, Merck, Germany).

Peptide synthesis

The GSH analogues were synthesized manually using Fmoc-chemistry and by machine using *tert*-Boc-chemistry [30,31]. For the manual peptide synthesis we used Gly–Wang resin or on Rink Amide MBHA resin. Couplings of Fmoc protected amino acids were carried out in a stepwise manner using the standard TBTU and HOBt activation in DMF. The peptides were removed from the resin and simultaneously deprotected with TFA in the presence of scavengers, water 2% (v/v), EDT 2% (v/v) and TIS 2.5% (v/v) for 90 min at room temperature.

The automated synthesis of UPF peptides was carried out in a stepwise manner in a 0.1 mmol scale on an Applied Biosystem Model 431A peptide synthesizer on a solid support using DCC/HOBt activation strategy. *tert*-Butyloxycarbonyl (*tert*-Boc) amino acids were coupled as hydroxybenzotriazole esters to a phenylacetamidomethyl-resin (0.6 mmol/g, Novabiochem, Switzerland) to achieve the C-terminal free carboxylic acid or to a *p*-methylbenzylhydryl-amine (MBHA) resin (1.1 mmol/g, Bachem, Switzerland) to obtain C-terminally amidated peptides. The peptides were finally cleaved from the resin with liquid HF at 0°C for 30 min. Deprotection of the side chains, cleavage of the peptides and purification on HPLC have been described in detail earlier [32].

The purity of the peptides was >99% as demonstrated by HPLC on an analytical Nucleosil 120–3 C18 reversed-phase column (0.4 × 10 cm). The molecular masses of the peptides were determined by a matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass-spectrometry (Voyager DE Pro, Applied Biosystems) and the calculated values were obtained in each case.

Hydroxyl radical scavenging ability

The hydroxyl radical scavenging ability of UPF peptides was measured as described by Barreto et al. using THA as a chemical dosimeter [33]. 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.

DPPH radical scavenging assay

The scavenging effect of the peptides on DPPH radical was measured spectrophotometrically (Jenway 6405 UV/Vis spectrophotometer, Jenway Ltd., England). 0.1 ml of peptide solution in saline (0.9% NaCl) in a concentration range from 2.5 to 200 μM was added to 0.1 ml of 0.1 mM $DPPH^{\cdot}$ in 95% ethanol. The mixture was shaken. 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 $DPPH^{\cdot}$ scavenging activity. The percentage of remaining $DPPH^{\cdot}$ against the peptide concentration was plotted to obtain the amount of antioxidant necessary to decrease by 50% the initial

DPPH$^{\cdot}$ 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 $1/EC_{50}T_{EC50}$ [34,35].

Dimerization of UPF peptides

The dimerization rate of UPF1 and UPF17 as the representatives of UPF peptides with the two different GSH backbones containing γ- and α-glutamate, respectively, was followed in water and in saline (0.9% NaCl) solutions. 1 mM solutions of peptides were kept at room temperature and at certain time points during 14 days, 100 μl samples were taken and analysed on analytical HPLC (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 of 2 ml/min. The wavelength of peak detection was 220 nm. Peak areas were calculated by the ChemStation software of Hewlett Packard HPLC system (model 1100). The quantities of reduced and oxidized forms of studied analogues in the sample were expressed in percents. Summarized areas of the peaks of monomeric and dimeric forms of UPF peptides were constant and were considered as 100% through all experiments. Fractions were collected and molecular masses of peptides (monomeric and dimeric forms) were determined by a MALDI-TOF mass-spectrometry (Voyager DE Pro, Applied Biosystems).

Cell culture

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).

K562 human erythroleukemia cells (a kind gift from Dr T. Land, Department of Neurochemistry and Neurotoxicology, Stockholm University, Stockholm, Sweden) 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 and 10^6 cells/ml.

Effects of UPF1, UPF6, UPF17, UPF19 on viability of K562 cells

CellTiter-GloTM Luminescent Cell Viability Assay (Promega, Madison, WI, USA) was used to estimate 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: 37,500 cells in 0.25 ml medium. Then 5 μl of drug stock solution was added. Stock solution concentrations were as follows: 10 mM in water for UPF1, UPF6, UPF17, UPF19 and 1 mM in DMSO:H_2O (10/90) for MTX. The final concentrations were as follows: 200 μM for UPFs, 20 μM for MTX. After 24 h of exposure in the cell culture incubator, the plate was removed from the incubator and equilibrated to room temperature for approximately 30 min. Then 0.25 ml CellTiter-Glo™ reagent (prepared according to manufacturer's instructions) was added to each well. After 10 min of gentle shaking, 400 μl of the content from each well was transferred to respective well on a white polypropylene LumiNunc™ plate (Nunc A/S, Roskilde, Denmark) and the luminescence was recorded on a dual-scanning microplate spectrofluorometer SPECTRAmax® GEMINI XS from Molecular Devices (Sunnyvale, CA, USA).

Effects of UPF1, UPF6, UPF17 and UPF19 on the membrane integrity of K562 cells

CytoTox-One™ Homogenous Membrane Integrity Assay (Promega) 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 lactate dehydrogenase (LDH) release from cells with a damaged membrane. About 15 ml of cells ($\leq 10^6$ cells/ml) were centrifuged for 5 min at 500*g*. The cell pellet was washed twice with 10 ml HEPES-buffered Krebs-Ringer solution (HKR: 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 1×10^6 cells/ml (counted with hemacytometer). About 200 μl of this suspension was transferred to a vial which already contained 200 μl of peptide or Triton X-100 solution in HKR. The final concentrations were as follows: 100 μM for UPFs, 10 μM for 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). About 100 μl of CytoTox-One™ Reagent (prepared according to the manufacturer's instructions) was added to each well. After 10 min of incubation at room temperature, 50 μl of stop solution was added to each well and the fluorescence was recorded on a dual-scanning microplate spectrofluorometer SPECTRAmax® GEMINI XS from Molecular Devices (Sunnyvale) using following wavelengths: 560 nm excitation and 590 nm emission.

Data analysis

All the data was analysed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA, USA). The results in the tables are

presented as the mean ± standard error of the mean (SEM).

Results

Solubility of peptides

All the synthesized peptides were readily soluble in water solutions up to 1 mM, except the biotinylated analogue, UPF7. UPF7 was designed to study intracellular interactions of UPF1 but hydrophobic biotinyl moiety significantly decreased solubility of the analogue in water solutions and therefore UPF7 was not used in the following experiments.

Hydroxyl radical scavenging assay

The hydroxyl radical scavenging assay was used as the first estimation criteria of the antioxidative activity of the designed peptides. The hydroxyl radicals were generated via reaction between Cu^{2+} and H_2O_2 and detected with a fluorescence method using THA as a probe. The EC_{50} of the hydroxyl radical scavenging reaction for UPF peptides are shown in Table I. The results showed that all designed peptidic molecules were remarkably stronger hydroxyl radical scavengers, than GSH (Table I). Peptides with γ-peptide linkage in backbone showed the EC_{50} between 17 and 35 μM compare to GSH 1231.0 ± 311.8 μM, respectively. The substitution of the all or only N-terminal L-amino acid to D-enantiomer did not change the hydroxyl radical scavenging abilities. The comparison of radical scavenging properties of free acid and amidated form in the next pairs of peptides—UPF1 and UPF 8, UPF 14 and UPF10, UPF6 and UPF27, UPF 17 and UPF 25 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 not only SH group is involved in radical depletion reaction.

Most powerful antioxidants in the hydroxyl radical scavenging assay turned out to be the sequences were γ-glutamate residue was replaced with α-glutamate residue (UPF17–UPF25) (Figure 1b). These analogues showed EC_{50} of scavenging reaction in the submicromolar range (EC_{50} was between 30 and 50 nM) and peptide solution with concentration 1 μM achieved approximately 80% from the maximal inhibiting effect (Figure 2). Exact EC_{50} measurements in case of α-glutamate 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 extremely small amount of peptides.

DPPH radical scavenging assay

This assay is based on the reduction of $DPPH^{\cdot}$, a stable free radical. $DPPH^{\cdot}$ has a strong absorption at 517 nm and upon reduction by a free radical scavenging antioxidant, this absorption is decreased. We studied the effects of the most interesting GSH analogues and we found that they all exhibited free radical scavenging activity against the stable free radical DPPH. Table II summarizes the EC_{50} concentrations, the time needed to reach the steady state EC_{50} concentrations (T_{EC50}) and antiradical efficiency (AE, $1/EC_{50}T_{EC50}$). Compared to GSH all designed molecules have similar approximately 1.2-fold greater EC_{50} concentrations required to scavenge 50% of the DPPH stable free radical. Although GSH was a slightly better DPPH radical scavenger by

Table I. Library of designed and synthesized GSH analogues and their hydroxyl radical scavenging ability.

Nr.	Sequence	MW	EC_{50} ± SEM (μM)
UPF1	H_2N-Tyr(Me)—(γ-Glu)—Cys—Gly—COOH	484.5	20.5 ± 1.3
UPF2	H_2N-(γ-Glu)—Cys—Gly—Tyr(Me)—COOH	484.5	19.8 ± 0.8
UPF5	H_2N-D-Asp—(γ-Glu)—Cys—Gly—COOH	421.4	34.7 ± 0.9
UPF6	H_2N-D-Ser—(γ-Glu)—Cys—Gly—COOH	394.4	21.2 ± 0.4
UPF7	Biotinyl-Tyr(Me)—(γ-Glu)—Cys—Gly—COOH	710.5	ND*
UPF8	H_2N-Tyr(Me)—(γ-Glu)—Cys—Gly—$CONH_2$	483.5	24.4 ± 0.4
UPF10	H_2N-D-Tyr(Me)—D-(γ-Glu)—D-Cys—Gly—$CONH_2$	483.5	25.5 ± 0.6
UPF14	H_2N-D-Tyr(Me)—D-(γ-Glu)—D-Cys—Gly—COOH	484.5	19.6 ± 0.8
UPF15	H_2N-Tyr—(γ-Glu)—Cys—Gly—COOH	469.4	19.0 ± 1
UPF16	H_2N-Ser—(γ-Glu)—Cys—Gly—COOH	394.4	17.3 ± 1.1
UPF17	H_2N-Tyr(Me)—Glu—Cys—Gly—COOH	484.5	0.038 ± 0.003
UPF18	H_2N-D-Tyr(Me)—Glu—Cys—Gly—COOH	484.5	0.044 ± 0.007
UPF19	H_2N-D-Ser—Glu—Cys—Gly—COOH	394.4	0.031 ± 0.004
UPF24	H_2N-Ser—Glu—Cys—Gly—COOH	394.4	0.046 ± 0.003
UPF25	H_2N-Tyr(Me)—Glu—Cys—Gly—$CONH_2$	483.5	0.032 ± 0.006
UPF26	H_2N-Tyr(Me)—Glu—Ser—Gly—COOH	468.5	21.1 ± 0.9
UPF27	H_2N-D-Ser—(γ-Glu)—Cys—Gly—$CONH_2$	393.4	22.1 ± 0.9
GSH	(γ-Glu)—Cys—Gly—COOH	307.3	1231.0 ± 311.8

* ND, not determined.

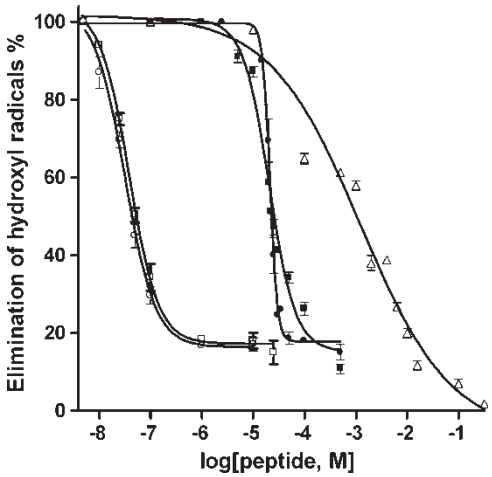

Figure 2. Concentration dependent hydroxyl radical scavenging effects of UPF1 (■), UPF6 (●), UPF17 (□), UPF19 (○) and GSH (Δ) *in vitro*.

comparing EC_{50} values, all designed molecules achieved their steady state EC_{50} with shorter time (T_{EC50}) and thereby gave better antiradical efficiency than GSH. Peptides with α-glutamate in backbone (UPF17 and UPF19) gave the highest antiradical efficiencies, whereas UPF17 was the best. The T_{EC50} of GSH was ten times higher than for UPF17 and the antiradical efficiency of UPF17 was 8.3-fold greater than GSH. Comparison of T_{EC50} in pairs of peptides UPF1 and UPF8, UPF17 and UPF25 revealed that amidated peptides need about 2-fold more time to reach steady state than the similar free acid peptides, T_{EC50} 10 and 20, 4 and 7 min, respectively.

Dimerization of UPF peptides

We studied the rate of dimerization of the selected UPF peptides (UPF1 and UPF17) in water and in saline solutions to clarify the state of molecules in our other experiments. In water and physiological solution analogues showed different dimerization rate at 1 mM concentration (Figure 3). Performing MALDI-TOF analysis, we found the first signs of dimerization in the solution on the 2nd-day of incubation. For all peptides studied dimerization occurred quicker 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 dimers in water solution whereas the same amount of dimer in saline was detected on the 7th-day.

Table II. DPPH stable free radical scavenging capacity—antiradical efficiencies (AE). AE is $1/EC_{50}T_{EC50}$.

Compounds	EC_{50} (μM)	T_{EC50} (min)	AE ($\times 10^{-3}$)
GSH	23.6 ± 2.1	40	1.06
UPF1	28.1 ± 1.6	10	3.56
UPF6	29.0 ± 1.5	21	1.64
UPF17	28.3 ± 1.7	4	8.83
UPF19	27.0 ± 0.9	9	4.12
UPF8 (UPF1 amide)	29.3 ± 2.0	20	1.71
UPF25 (UPF17 amide)	36.4 ± 1.8	7	3.89

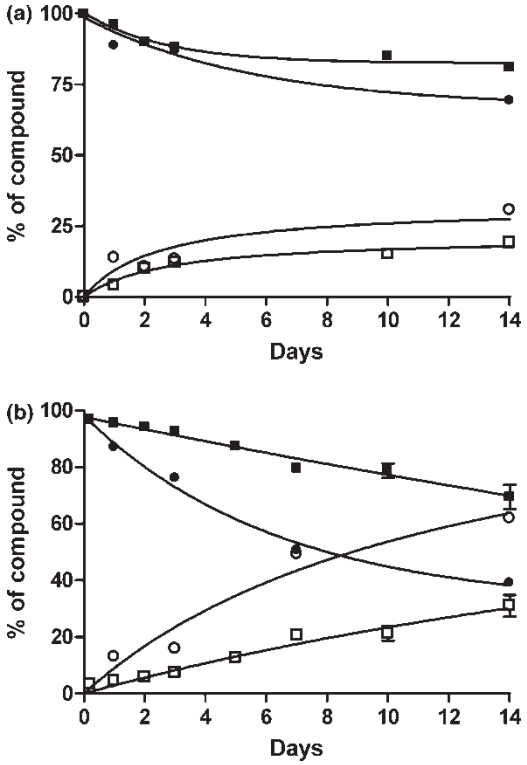

Figure 3. Dimerization of UPF1 and UPF17 in water and in 0.9% NaCl solution at room temperature. Content of monomeric (●) and dimeric (○) form of UPF1 and of monomeric (■) and dimeric (□) form of UPF17 in water (a) and in 0.9% NaCl solution (b).

Effects of UPF peptides on the viability and membrane integrity of K562 erythroleukemia cells

We have previously shown that UPF1 did not affect viability of primary cerebellar granule cells at 100 μM concentration. To study further toxic effects of UPF peptides we choose to work with K562 human erythroleukemia cells. K562 cells are widely used in differentiation studies and are often selected for studies of anticancer drugs. We compared the effects of different UPF peptides on the viability and on the membrane integrity of K562 cells (Figure 4). In the viability experiments, we compared effects of 200 μM solutions of peptides and 20 μM MTX on K562 cells after 24 h. UPF1, UPF6, UPF17 and UPF19 did not show any toxic effects on the viability of K562 cells, whereas only 35% of cells survived MTX treatment.

Furthermore, we studied the effects of the same set of peptides on the integrity of K562 cells membrane in LDH leakage assays. In these experiments we compared the effects of UPF peptides with the effect of MAP.

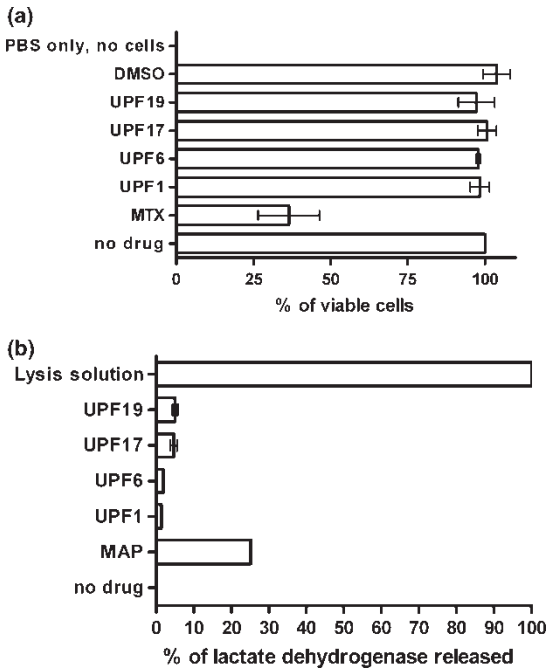

Figure 4. The toxicity test. The influence of UPF1, UPF6, UPF17 and UPF19 to K562 human erythroleucemia cells. (a) UPF 200 μM, MXT 20 μM; (b) UPF 100 μM, MAP 10 μM.

The effects of MAP have been previously studied [36]. When the background fluorescence ("no drug") was taken as 0% and the Triton X-100-induced signal was taken as 100%, the total cellular LDH leaked from K562 cells after MAP treatment was 25%. None of the UPF peptides caused significant LDH leakage from K562 cells. Interestingly, UPF17 and UPF19 disturbed membranes more than peptides with γ-peptide linkage (UPF1 and UPF6).

Discussion

Various low-molecular antioxidants, including melatonin, carvedilol and its metabolite SB211475, have a methoxy moiety in their aromatic structures. It has been shown that the methoxy group increases the antioxidative activity of different compounds [37]. To study the effect of the methoxy moiety we first designed the tetrapeptide UPF1, where we added *O*-methyl-L-tyrosine to the N-terminus of GSH. We have shown that this change increased the hydroxyl radical scavenging ability 60-fold compared to GSH itself *in vitro* [27]. Taken that into account and to investigate the structure-related effects on activities of UPF1 we prepared a series of UPF peptides (Table I). Different amino acids were added to a GSH molecule via a peptide bond, resulting in tetrapeptides. Mainly, the additional unit was added to the N-terminus, but positioning in the C-terminus was also investigated (UPF2). Part of the library was synthesized so that α-glutamate was used instead of γ-glutamate (UPF17–UPF25). In the case of several peptides, all L-amino acids (UPF10 and 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 sulphydryl group.

The ability of GSH and UPFs to scavenge hydroxyl radicals suggests that UPFs are electron donors, which can react with free radicals to convert them to more stable products and terminate a radical chain reaction. Radical scavenging assays revealed very interesting results—the addition of different moieties to the N- or C-terminus of GSH, the exchange of L-amino acids to D-forms, the amidation of peptides or even the change of Cys to Ser did not influence drastically hydroxyl radical scavenging properties of peptides compared to UPF1. All these analogues had EC_{50} values between 17 and 35 μM whereas the change of γ-glutamate to the α-glutamate drastically decreased EC_{50} by approximately 100 times. 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. When comparing EC_{50} values of hydroxyl radical elimination measured by THA method, UPF peptides with α-glutamate in their backbone exceeded even the respective property of an exhaustively tested antioxidant melatonin (*N*-acetyl-5-methoxytryptamine; EC_{50} 11.4 ± 1 μM) [38].

We also tested the ability of GSH and UPF peptides to scavenge DPPH radicals. We determined the scavenging activity of UPF1, UPF6, UPF8, UPF17, UPF19 and UPF25 in cell free systems using $DPPH^{\cdot}$ and compared their activity with that of GSH. Based on the T_{EC50} Sanchez-Moreno et al. classified the kinetic behaviour of the antioxidant compound as follows: <5 min (rapid), 5–30 min (intermediate), and >30 min (slow) [34]. According to this classification UPF17 was rapid; UPF1, UPF6, UPF8, UPF19 and UPF25 were intermediate; and GSH was a slow antioxidant. The comparison of times required for half reaction, T_{EC50}, of the tested compounds showed that T_{EC50} for UPF1 was 4-fold and for UPF6 2-fold shorter than that required for GSH. T_{EC50} for UPF17 and UPF19 were 2.5-fold shorter than UPF1 and UPF6, respectively. The DPPH radical was scavenged by GSH and UPF peptides through donation of hydrogen to form the stable DPPH-H. The results showed that the methoxy group makes the compounds more likely to react with free radicals [37].

As expected only reduced forms of GSH and UPF peptides can react with other compounds/radicals. Previously we have studied the stability of UPF peptides by capillary electrophoresis [39,40]. All active analogues showed slow dimerization kinetics in water and saline solution. We found that dimerization is slow (days) process, but occurs faster in presence of salt.

We also studied the toxicity of designed peptides. UPF peptides did not show any influence on the viability of K562 cells even at 200 μM concentration. Also they practically did not disturb the plasma membrane structure of the cells. For disturbance of membranes, our designed peptides are too short, but again, slightly more effect had the peptides which included α-peptide bond in the structure. At the same time, addition of more hydrophobic moiety into the sequence did not influence the membrane perturbance. The non-toxic influence of UPF peptides is also confirmed with the results of previous work: UPF1 was not toxic to nervous tissue up to 100 μM concentration [27].

The action mechanism of UPF peptides still need to be clarified. In addition to the proven qualities like the free radical scavenging effect as well as the modulation of the G proteins in frontocortical membrane, their ability to increase the intracellular GSH level and normalize the GSH/GSSG ratio will be investigated in the near future. In conclusion, we have designed and synthesized the library of novel nontoxic antioxidants, which showed very potent antioxidativity when compared with GSH and they have impact both as potent antioxidants and provide promising leads for design of powerful non-peptidic antioxidants for correction of oxidative stress-driven events.

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