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Research paper

Glutathione salts of O,O-diorganyl dithiophosphoric acids: Synthesis and study as redox modulating and antiproliferative compounds

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ABSTRACT

Reactions of glutathione (GSH) with O,O-diorganyl dithiophosphoric acids (DTPA) were studied to develop bioactive derivatives of GSH. Effective coupling reaction of GSH with DTPA was proposed to produce the ammonium dithiophosphates (GSH–DTPA) between the NH_2 group in γ-glutamyl residue of GSH and the SH group in DTPA. A series of the GSH–DTPA salts based on O-alkyl or O-monoterpenyl substituted DTPA were synthesized. Enhanced radical scavenging activity of the GSH–DTPA over GSH was established with the use of DPPH assay and improved fluorescent assay which utilizes Co/H_2O_2 Fenton-like reaction. Similarly to GSH, the dithiophosphates induced both pro- and antioxidant effects in vitro attributed to different cellular availability of the compounds. Whereas extracellularly applied GSH greatly stimulated proliferation of cancer cells (PC-3, vinblastine-resistant MCF-7 cells), the GSH–DTPA exhibited antiproliferative activity, which was pronounced for the O-menthyl and O-isopinocampheolyl substituted compounds **3d** and **3e** ($IC_{50} \geq 1$ μM). Our results show that the GSH–DTPA are promising redox modulating and antiproliferative compounds. The approach proposed can be extended to modification and improvement of bioactivity of various natural and synthetic peptides.

1. Introduction

L-γ-Glutamyl-L-cysteinyl-glycine (glutathione, GSH) is the prevailing antioxidant oligopeptide in mammals, which plays a crucial role in non-specific and enzyme-assisted defense of living cells from oxygen radicals, detoxification of xenobiotics, maintenance and regulation of the redox homeostasis in cells [1–4].

The association of GSH deficiency with a variety of human metabolic, degenerative, aging-related diseases [4] and viral infection [3] is well established. At the same time, elevated GSH level is often involved in tumor resistance and progression [5]. GSH, its analogues and metabolizing enzymes attract considerable interest in biomedical and pharmacological research. Most enzymes of the GSH metabolism, e.g. catalyzing biosynthesis of GSH (γ-glutamyl transferase), nucleophilic addition reactions of the thiol group (glutathione-*S*-transferase), methylglyoxal detoxification (glyoxalase) and redox reactions (glutathione reductase, glutathione peroxidase) are established pharmacotherapeutic targets [1,6].

A number of synthetic approaches to developing enzyme effectors based on the tripeptide GSH have been proposed to date. Main strategies for generation of the bioactive analogues and derivatives of GSH, summarized by Lucente et al. [1], include replacement of one or more amino acids in the tripeptide backbone with artificial analogues (*D*-, N-methyl-, α-methyl-glutamic acid, α-methyl-L-cysteine, β-alanine) as well as modification of the SH group of cysteine in order to produce both reversible and irreversible inhibitors of GSH-metabolizing enzymes (see [1] and references within). Some other approaches in GSH chemistry proposed are aimed at the GSH derivatives with increased biological stability, e.g. resistance to blood γ-glutamyl transpeptidase, by means of esterification of the carboxyl functions [6] and cyclization of the GSH molecule [1].

Burg and Mulder summarized the state of the art in developing derivatives and analogues of GSH with antiproliferative activity and studying their capacity to overcome cancer drug resistance associated with GSH-dependent enzymes [6]. These enzymes are glutathione *S*-transferases catalyzing GSH conjugation to harmful electrophilic compounds, including carcinogens and anticancer drugs, DNA-dependent protein kinase (PI 3-kinase family) involved in repairing double-strand breaks in DNA as well as glyoxalase system (glyoxalases I and II) participating in elimination of cytotoxic α-oxoaldehydes [6]. Some examples of effective inhibitors of these enzymes include *S*-alkyl derivatives of γ-glutamyl-2-aminoadipic acid analogue, GSH derivatives

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modified with phenyl and phosphonic groups, *S*-*p*-bromobenzyl-glutathione (see [6] and references within).

Considerable efforts are being made to utilize therapeutic potential of GSH and its precursors as intracellular antioxidants and regulators of the redox state in mammalian cells. Involvement of the oxidative stress and concomitant GSH depletion was shown for many disorders including those affecting skin, leaver, pulmonary and ocular tissues as well as preeclampsia, myocardial ischemia, neurodegenerative diseases, and decreased immune function [4,7]. Restoring balance between the reduced form of glutathione (GSH) and the oxidized one (GSSG) by using precursor compounds helps to diminish the oxidative injury and its pathological consequences [2,4,7].

Since a relatively low pharmacotherapeutic potential of GSH per se upon both oral and parenteral administration [4], prodrugs and derivatives of GSH with improved pharmacokinetic properties are being discovered. These compounds are particularly targeted at antiviral therapy against retroviruses, including HIV, influenza, rhinovirus, herpes simplex virus [3]. *N*-acetyl-cysteine (NAC), β-mercaptoethylamine derivative of NAC, GSH monoethylester, *S*-acetylglutathione, and *N*-butanoyl GSH were shown to act as prodrugs which increase intracellular level of GSH and other thiols and exert antiviral effects (see [3] and references within).

S-trityl-L-cysteine derivatives [8] and alkyl chain modified GSH derivatives coupled through *N*-terminal NH_2 group [9] with increased inhibitory activity towards hepatitis C and herpes simplex viruses, respectively, were synthesized. Antiviral activity of the GSH derivatives could result from both direct inhibition of the viral replication at different stages and immunomodulatory activity of the compounds [3,10]. *S*-acyl glutathione derivatives with fatty acids, such as *S*-lauroylglutathione and *S*-palmitoleoylglutathione, were recently developed as potential therapeutics in neurodegenerative diseases. These compounds have increased cellular availability, protect SH-SY5Y cells and cholinergic neurons against amyloid-induced oxidative damage and apoptosis via regeneration of intracellular pool of GSH [11,12].

In spite of a number of bioactive derivatives of GSH reported there is, however, the lack of the approved drugs which encourages development of alternative approaches to modification of GSH. To date, different strategies to modify peptide based drugs have been elaborated [13]. Organophosphorus compounds are promising modifiers for natural and synthetic peptides, which allow for improvement of their physicochemical and biological properties. Among them, dithiophosphoric acids (DTPA) are insufficiently explored compounds with previously established insecticide [14], antimicrobial activities [15–17], and which are relatively low-toxic for warm-blooded animals in contrast to phosphoric acid esters [14].

Different DTPA derivatives with natural alcohols, phenols and monoterpenes were earlier synthesized [15–18]. These DTPA, being a huge lipophilic anion, can form ammonium dithiophosphates with hydrophilic amino acids as a result of quaternization of the most basic nitrogen atom by the acidic SH group of DTPA. It was assumed that the reaction of O,O-diorganyl DTPA with GSH will improve properties of the tripeptide and generate new bioactive compounds. We provide proof-of-concept of this chemical approach and study effects of the dithiophosphoryl groups on antioxidant and antiproliferative properties of the GSH derivatives.

2. Material and methods

2.1. Materials

Tetramethylrhodamine, ethyl ester, 2′,7′-dichlorofluorescin diacetate, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2′,7′-dichlorofluorescin diacetate, 2,2′-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich. BODIPY 581/591 C11 lipid peroxidation sensor was obtained from Thermo Fisher Scientific. Reduced glutathione (purity 98%) and oxidized glutathione (purity 95%) were purchased from Acros Organics.

(*S*)-(−)-menthol (purity 99.5%) was purchased from Acros Organics. (1*S*,2*S*,3*S*,5*R*)-(+)-isopinocampheol (purity 98%) was purchased from Sigma-Aldrich.

Materials for cell culturing were obtained from PAA Laboratories. Milli-Q grade water (Milli-Q® Advantage A10, Merck Millipore) was used to prepare buffers and solutions.

2.2. Synthesis of O,O-diorganyl dithiophosphoric acids

O,O-Dialkyl dithiophosphoric acids **2a** (δ_P 85.4 ppm), **2b** (δ_P 83 ppm) and **2c** (δ_P 86.4 ppm) were synthesized as described previously [19].

O,O-Bis[(−)-(1R,2S,5R)-2-isopropyl-5-methylcyclohex-1-yl] dithiophosphoric acid (**2d**) was synthesized by the reaction of tetraphosphorus decasulfide with (*S*)-(−)-menthol ($[\alpha]_D^{20}$–20.8°, c 1.035, C_6H_6), mp 61–63 °C, δ_P 81.3 ppm, C_6H_6) as reported recently [20].

O,O-Bis[(+)-(1*S*,2*S*,3*S*,5*R*)-trimethylbicyclo[3.1.1]hept-3-yl dithiophosphoric acid (**2e**) was synthesized as follows. Tetraphosphorus decasulfide (0.9 g, 8.3 mmol) was added portionwise under dry argon with stirring at 20 °C to the solution of (1*S*,2*S*,3*S*,5*R*)-(+)-isopinocampheol (1.7 g, 11 mmol) in 20 mL anhydrous benzene, and stirring was continued for 2 h at 50 °C. The mixture was stored at 20 °C overnight and filtered. The filtrate was evaporated at reduced pressure (0.5 mm Hg) at 40 °C for 1 h and then in vacuum (0.02 mm Hg) for 1 h to give **2e** (1.8 g, 77%). Acid **2e** was purified by use of a column chromatography (silica gel 0.060–0.200 μm, eluent − benzene). R_f 0.45 (hexane). n_D^{20} 1.5230. $[\alpha]_D^{22}$ +35.0° (c 1.0, C_6H_6). FTIR (cm^{-1}, liquid film): ν_{max} 2987_{st}, 2911_{st} (CH_3 as, s; CH_2 as, s), 2583_w (S–H, free), 2403_w (S–H $_{related}$), 1471_{st}, 1452_{st} δ (CH_3 as); 1385_m, 1368_m δ [$(CH_3)_2C$ gem. s], 1089_m [(P)O—C], 973_{st} (OC—C, C—C), 772_{st} (PO_2 as, s), 676_{st} (P = S), 521_m (P–S). ^{1}H NMR ($CDCl_3$) ppm: δ = 0.96 and 1.23 [two s, 12H, ($C^{9',10'}H_3)_2C$], 1.21 (d, 6H, $C^{8'}H_3$, $^3J_{HH}$ = 7.5 Hz), 1.97 (m, 4H, $C^{7'}H_2$), 2.24 (m, 2H, $C^{2'}H$), 2.38 (m, 4H, $C^{6'}H_2$), 2.62 (m, 2H, $C^{3'}H$), 4.96 (m, 2H, P-O$C^{1'}$H). ^{31}P-{^{1}H} NMR (benzene) ppm: δ = 85.1. Anal. Calcd. for $C_{20}H_{35}O_2PS_2$: C, 59.67; H, 8.76; P, 7.69; S, 15.93. Found: C, 59.98; H, 8.33; P, 7.58; S, 15.73%.

2.3. Synthesis of glutathione salts of dithiophosphoric acids (*3a–3e*)

2.3.1. General procedure

Dithiophosphoric acid **2** (0.81 mmol) was added drope wise under dry argon with stirring at 20 °C to the solution of glutathione **1** (0.81 mmol) in 15 mL anhydrous ethanol, and stirring was continued for 1.5 h at 50 °C. The mixture was filtered and the filtrate was evaporated at reduced pressure (0.5 mm Hg) at 40 °C for 1 h and then in vacuum (0.02 mm Hg) to give **3**. Volatiles were removed under reduced pressure.

The synthesis of **3a–3e** is detailed in the Supplementary data.

2.3.2. Structure characterization

Melting points were determined on an electrothermal (variable heater) melting point apparatus and are uncorrected. The optical rotations recorded on a Perkin-Elmer 341 polarimeter (Norwalk, CT, USA) with a pathlength 55.2 mm using the λ = 589 nm of *D*-line of sodium. FTIR spectra were obtained in film with a Bruker Tensor 27 infrared spectrophotometer (Bruker BioSpin AG, Fällanden, Switzerland) (400–4000 cm^{-1}) and expressed in cm^{-1}, δ = the deformation vibration, st = strong, w = weak, m = medium vibrations, s – symmetric and as − asymmetric vibrations; gem – geminal. The ^{31}P NMR spectra were taken on a Bruker Avance-400 (161.9 MHz) instruments (Bruker BioSpin AG, Fällanden, Switzerland) in ethanol with 85% H_3PO_4 as an external reference. The ^{1}H (400 MHz), ^{13}C and ^{13}C-{^{1}H} (100.6 MHz) spectra were run at ambient temperature on a Bruker Avance (III) 400 instruments (Bruker BioSpin AG, Fallanden, Switzerland) in acetone-d_6. Chemical shifts (δ are given in parts per million (ppm) relative to

residual resonance of solvents. Coupling constants (*J*) are given in Hertz (Hz). Characterization of the multiplicities of signals: s = singlet, d = doublet, t = triplet, q = quartet, sept = septet, m = multiplet, br = broad. Mass spectra ESI were determined on a Bruker Compass DataAnalysis 4.0.

2.4. Study of antiradical activity

2.4.1. Fluorescent detection of Co/H_2O_2 reaction

Stock solution of cobalt (II) chloride was prepared by dissolving sample in deionized water. Stock solution of hydrogen peroxide (H_2O_2) was prepared by dilution 30% H_2O_2 in deionized water; H_2O_2 concentration was determined spectrophotometrically at λ = 240 nm on a LAMBDA-35 spectrophotometer (Perkin Elmer) using extinction coefficient of 43.6 M^{-1} cm^{-1}.

Cobalt chloride and H_2O_2 were mixed in PBS (pH 7.4) to obtain final concentrations of 0.2–1.0 mM (Co) and 2–22 mM (H_2O_2). 2′,7′-dichlorofluorescin diacetate (DCF-DA) was then added at final concentration of 5 μM to detect oxygen radicals produced. The reaction was carried out at ambient temperature in 96-well plate in the presence of an effector compound at serial dilutions or without effectors (reference). The fluorescence intensity was registered at λ_{ex} = 488 nm and λ_{em} = 535 nm kinetically for 60 min on an Infinite M200 PRO microplate analyzer (TECAN).

Sigmoidal concentration-response curves for effector compounds relative to the reference reaction (assumed as 100%) were constructed and half-maximal effective (inhibitory) concentrations (EC_{50}) were calculated from these curves using OriginPro 8.0 software.

2.4.2. DPPH assay

DPPH assay was carried out as described previously [21]. Briefly, 2,2-diphenyl-1-picrylhydrazyl (DPPH) was dissolved in methanol at stock concentration of 4 mM. The reaction solution in PBS contained DPPH at final concentration of 250 μM and an effector compound at serial dilutions. The mixture was kept for 60 min at room temperature. The optical absorbance of the solution was detected in 96-well plate at wavelength of λ = 515 nm on an Infinite M200 PRO microplate analyzer (TECAN). The background signal at reference wavelength of λ = 720 nm was subtracted from the signal. The concentration-response curves and EC_{50} values were obtained as described in the paragraph 2.4.1.

2.5. Evaluation of effects on mammalian cells

2.5.1. Cell lines and cultures

Human breast cancer (MCF-7) and prostate adenocarcinoma (PC-3) cell lines (ATCC) were used. Postsurgical full-thickness skin fragments of healthy donors were obtained from the Republican Clinical Hospital (Kazan, Russia). Human skin fibroblasts (HSF) were used under a protocol approved by the Institutional Ethical Review Board of the Kazan Federal University (Protocol #1, 30 Apr 2015). Skin explants were aseptically minced with fine scissors, transferred into the culture flask and cultured as detailed below to allow HSF to freely migrate from the tissues onto the flask surface.

Vinblastin-resistant MCF-7 (MCF-7/Vinb) cells were obtained by a series of passaging of MCF-7 cells with gradually increased concentrations of vinblastine. The MCF-7/Vinb cells were maintained by culturing them with 1 nM supplement of vinblastine.

MCF-7, MCF-7/Vinb and PC-3 cells were cultured aseptically in DMEM containing 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/mL streptomycin at 37 °C in humidified air atmosphere with 5% CO_2. HSFs were cultured in the same conditions but in α-MEM medium with the same supplements.

2.5.2. MTT assay

The cytotoxicity of the compounds synthesized was evaluated by means of the MTT assay as follows. The grown cells were washed with Hank's balanced salt solution (HBSS) and collected from culture flask by treating with trypsin–EDTA dissociation solution. The suspended cells were seeded in a 96-well plate at a density of 1000 cells per well in the culture medium and cultured overnight. Serially diluted solutions of the compounds were prepared aseptically from stock solutions and sterile water. The culture medium was replaced by a fresh one and the compounds were added to the cells at concentrations from 60 nM to 2.5 mM. Sterile water was added instead of compounds as a control. The cells were cultured with the compounds under standard conditions for 72 h; then the medium was replaced by a fresh one containing the MTT reagent (0.5 mg/mL). The cells were additionally cultured for 3 h to allow them to reduce MTT into water insoluble formazan followed by the medium discarding and formazan dissolution with 100 μL of DMSO per well. The optical absorbance of the formazan solution, which is proportional to the number of viable cells, was measured in each well using an Infinite M200 PRO microplate analyzer (Tecan) at the wavelength of 555 nm. The cell viability was calculated as a percentage of control cells grown without compounds (100% viability).

The data were fitted using 'dose response/sigmoidal' function (OriginPro 8 software) y = A1 + (A2-A1)/(1 + 10^ ((IC50-x)*p)), where y is viability (%), x is concentration, A1 is the bottom asymptote, A2 is the top asymptote (limited to 100%), IC50 is the half-maximal inhibitory concentration, p is Hill slope. The statistically significant difference was evaluated by Student's *t*-test with a significance level of $p < 0.05$.

2.5.3. Assessment of H_2O_2-induced oxidative burst

PC-3 cells were seeded in a 96-well plate and cultured until a confluent monolayer was formed. The cells were washed with HBSS, pre-stained with 20 μM DCFDA and rewashed with HBSS two times. Oxidative burst in the pre-stained cells was induced by treating them with 100 mM H_2O_2 in PBS for 1 h in the CO_2 incubator in the presence of a compound of interest at concentration from 10 μM to 10 mM. The fluorescent signal of the treated cells was registered on an Infinite M200 PRO microplate analyzer (TECAN) at λ_{ex} = 488 nm and λ_{ex} = 535 nm.

2.5.4. Analysis of transmembrane potential of mitochondria and membrane lipid peroxidation

PC-3 cells were seeded in a 6-well plate and cultured overnight in standard conditions. Next day the culture medium was replaced by a fresh one supplemented with the compounds at IC_{50} concentration. The cells were cultured in the presence of compounds for 48 h, and then collected from the plate by treating with trypsin–EDTA dissociation solution. The suspended cells (100 000 cells/mL) were stained with 100 nM tetramethylrhodamine ethyl ester (TMRE) in PBS for 30 min in CO_2-incubator upon moderate agitation. Distribution curves of the TMRE fluorescent signal in the cells and the mean channel fluorescence were obtained on a flow cytometer Guava Easy Cyte 8HT (Millipore) in the yellow channel.

For analysis of the membrane lipid peroxidation, the same procedure was carried out but the treated PC-3 cells were stained with 250 nM BODIPY 581/591 C11 lipid peroxidation sensor. Distribution curves of the BODIPY 581/591 C11 fluorescent signal in the cells and the mean channel fluorescence were obtained on a flow cytometer Guava Easy Cyte 8HT (Millipore) in the red channel.

3. Results

3.1. Synthesis and structure of glutathione salts of dithiophosphoric acids

The tripeptide glutathione (GSH, **1**) composed of *L*-glutamic acid, *L*-cysteine and glycine residues was reacted with the DTPA via α-NH_2 group of the γ-glutamyl moiety (Fig. 1). DTPA containing O-ethyl, O-butyl or O-isopropyl substituents (**2a, 2b, 2c,** respectively) were coupled to **1** to produce the ammonium dithiophosphates of GSH, e.g. the

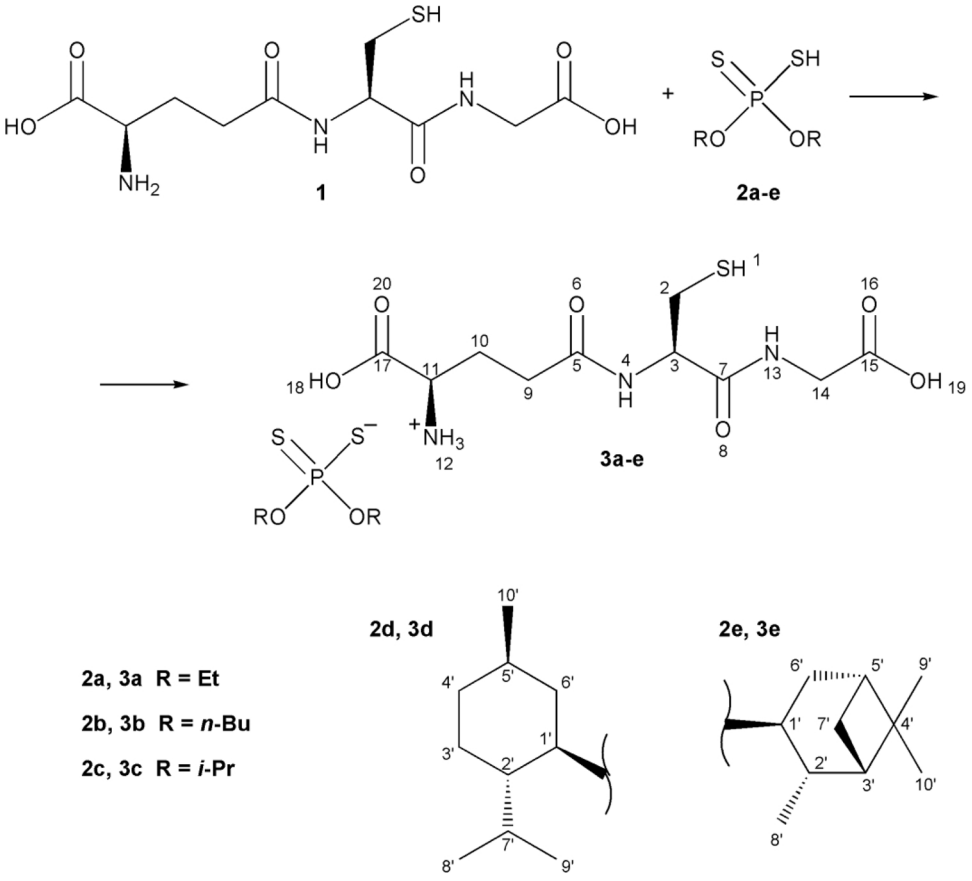


Fig. 1. Synthesis of glutathione ammonium salts of dithiophosphoric acids.

GSH–DTPA salts **3a, 3b, 3c**, respectively. Reactivity of DTPA **2a–2e** was found to depend on the polarity of solvent used. The reaction did not proceed in non-polar organic solvents, such as benzene and toluene, however was readily accomplished in more polar solvents such as ethanol.

Thus, the GSH–DTPA salts (**3a–3c**) were synthesized by reaction of the O-alkyl substituted DTPA (**2a–2c)** with GSH in ethanol at 60 °C for 2 h (Fig. 1). This procedure was extended to chiral DTPA pre-synthesized from monoterpenols, namely (*S*)-(–)-menthol (**2d**) and (1*S*,2*S*,3*S*,5*R*)-(+)-isopinocampheol (IPC, **2e**). Reactions of the compounds **2d** and **2e** with GSH were conducted under the same conditions (ethanol, 60 °C, 2 h) to produce corresponding ammonium dithiophosphates **3d** and **3e** with chiral centers.

The GSH–DTPA salts prepared were semisolids at ambient temperature; their characteristics are detailed in the 'Material and methods' section and Supplementary data. The compounds **3a–3e** were synthesized in high yields (83–88%). Optical rotation values of **3a–3e**, measured in acetone or ethanol, confirmed that the GSH moiety in these ammonium dithiophosphates maintains its optical activity.

The IR spectra of the compounds **3a–3e** contained strong characteristic bands at 1735–1730 cm^{-1} due to O═C–O stretching vibrations. Two bands of the stretching vibrations of the Amide I (NHC═O) and the Amide II (CNH) were observed at 1659–1653 and 1536–1538 cm^{-1}, respectively. Intense broad band at 3335–3318 cm^{-1} for **3a–3e** was assigned to the stretching vibrations of NH_3^+. ^{31}P–{^1H} NMR spectra of **3a–3c** in ethanol revealed signals at 112–105, which are characteristic of the ammonium dithiophosphates [22].

^1H NMR spectrum of **3a** in acetone-d_6 showed a triplet at 1.22 ppm assigned to the protons of two methyl groups, $C\underline{H}_3CH_2O$ ($^3J_{HH}$ = 7.1 Hz). Methylene protons of the two ethoxy groups of **3a** appeared as a doublet of quartets at 4.14 ppm, $CH_3C\underline{H}_2OP$, ($^3J_{PH}$ = 14.4 Hz). $C^{14}H_2$ protons of the GSH moiety of **3a** exhibited a singlet at 2.96 ppm ^{13}C–{^1H} NMR spectra of **3a–3e** in acetone-d_6 showed characteristic resonances of the carbon atoms in GSH. In particular, the carbon atoms of carbonyl and carboxyl groups of the GSH moiety of **3e** appeared as 4 singlets at 172.0 ($C^{17}OO$), 173.5 (NC^7 = O), 177.3 ($C^{15}OO$), and 179.1 (NC^5 = O), respectively. These ^1H and ^{13}C–{^1H} NMR data support that GSH preserves its intact structure in the ammonium salts with DTPA.

The GSH–DTPA salts synthesized were initially insoluble in aqueous solutions, and hence were dissolved in DMSO as a biocompatible solvent. Solutions of the DTPA and GSH–DTPA salts were stable except **2c** which, in contrast to the corresponding salt **3c**, was readily precipitated, thus hampering its evaluation. Stock solutions of the compounds in DMSO were dissolved in water or buffer solutions at micromolar concentrations for further analysis.

3.2. Antioxidant properties of GSH–DTPA salts

3.2.1. Establishment of fluorescent assay for Fenton reaction

Antioxidant properties of the GSH–DTPA salts were evaluated by their ability to scavenge DPPH (2,2-diphenyl-1-picrylhydrazyl) radical and inhibit prooxidant Fenton-like reactions using the DPPH assay and pre-optimized fluorescent assay based on Co/H_2O_2 system, respectively. The reaction of divalent transition metals Fe, Co, Cu, and Mn with H_2O_2 was accompanied by a gradual increase of fluorescence of the DCFDA probe (Fig. 2) due to generation of the oxygen radicals, such as hydroxyl radical. Phosphate-containing buffers were found to substantially promote these reactions, hence the analysis was performed in PBS which was compatible with the metals at concentrations used.

The fluorescent signal of DCFDA increased among the metals as follows: Mn, Fe, Cu and Co, where copper and especially cobalt ions generated oxygen radicals in a much more efficient way (Fig. 2A). To analyze GSH and its derivatives the Co/H_2O_2 system was selected due to its high prooxidant activity and biological relevance (see also the

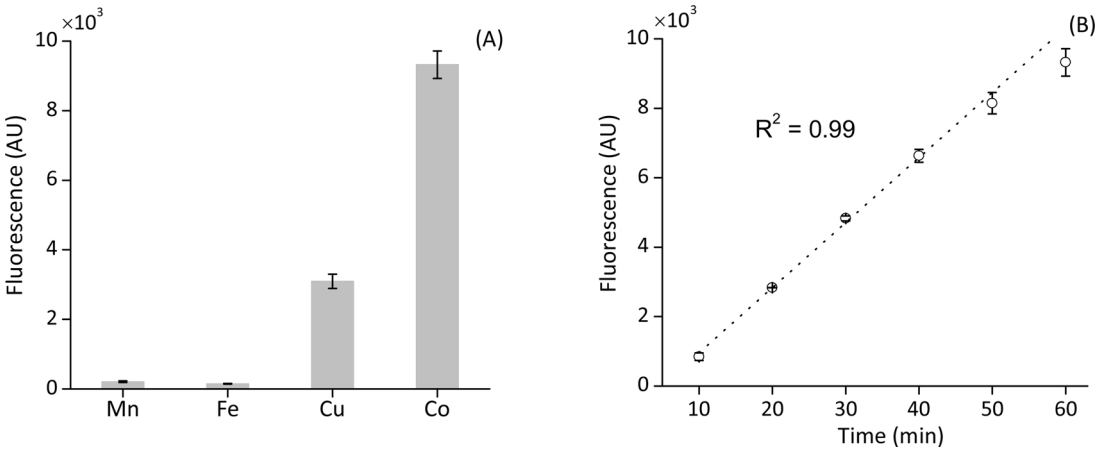


Fig. 2. (A) Generation of oxygen radicals in reactions of transition metals with hydrogen peroxide according to DCFDA fluorescence. (B) Kinetic curve of Co/H_2O_2 reaction. Microplate assay: λ_{ex} = 488 nm, λ_{em} = 535 nm. Solvent: PBS (pH 7.4), concentrations: DCFDA 5 μM, Co 0.23 mM, H_2O_2 21.6 mM. Mean ± SD (n = 3) are shown.

'Discussion' section). The microplate assay based on Co/H_2O_2 reaction was optimized as detailed in the 'Material and methods' section. The assay was characterized by a linear dependence of the signal on time for at least 60 min (Fig. 2B), ensuring a maximum reaction rate under these conditions.

3.2.2. Antiradical activity of GSH and GSH–DTPA salts

Relationships between inhibitory activity of the compounds and their concentrations in the reactions with DPPH and Co/H_2O_2 were presented in percentage of inhibitor-free reference reactions (100%) (Fig. 3), and corresponding half-maximal effective concentrations (EC_{50}) were calculated (Table 1). According to both assays, GSH in its reduced form possess comparable EC_{50} values of 248 μM (DPPH) and 544 μM (Co/H_2O_2). No effect of the oxidized glutathione (GSSG) on the DPPH probe was detected (Fig. 3C). In the Co/H_2O_2 assay, GSSG had somewhat lower EC_{50} value (365 μM) to that of GSH due to increased inhibitory activity of GSSG in the lower concentration range (Fig. 3).

In addition, the inhibitory curves of the compounds differed in their shape. As shown in Fig. 3A, GSH induced a gradual quenching of the DPPH radical with increase in concentration of the tripeptide (Fig. 3A), whereas a sharp inflection was observed in the Co/H_2O_2 reaction at a concentration of GSH above ~0.3 mM (Fig. 3B). In the higher concentration range, GSH suppressed the latter reaction more effectively than GSSG (Fig. 3B and D) presumably as a result of direct scavenging of oxygen radicals by the thiol group of the tripeptide. Altogether, these results suggest that GSH reacts with the DPPH probe through the thiol group, whereas in the Fenton reaction GSH shows a more complicated effect attributed to its different functional centers as will be discussed below.

The O-alkyl and O-monoterpenyl substituted DTPA **2a, 2b, 2d, 2e**

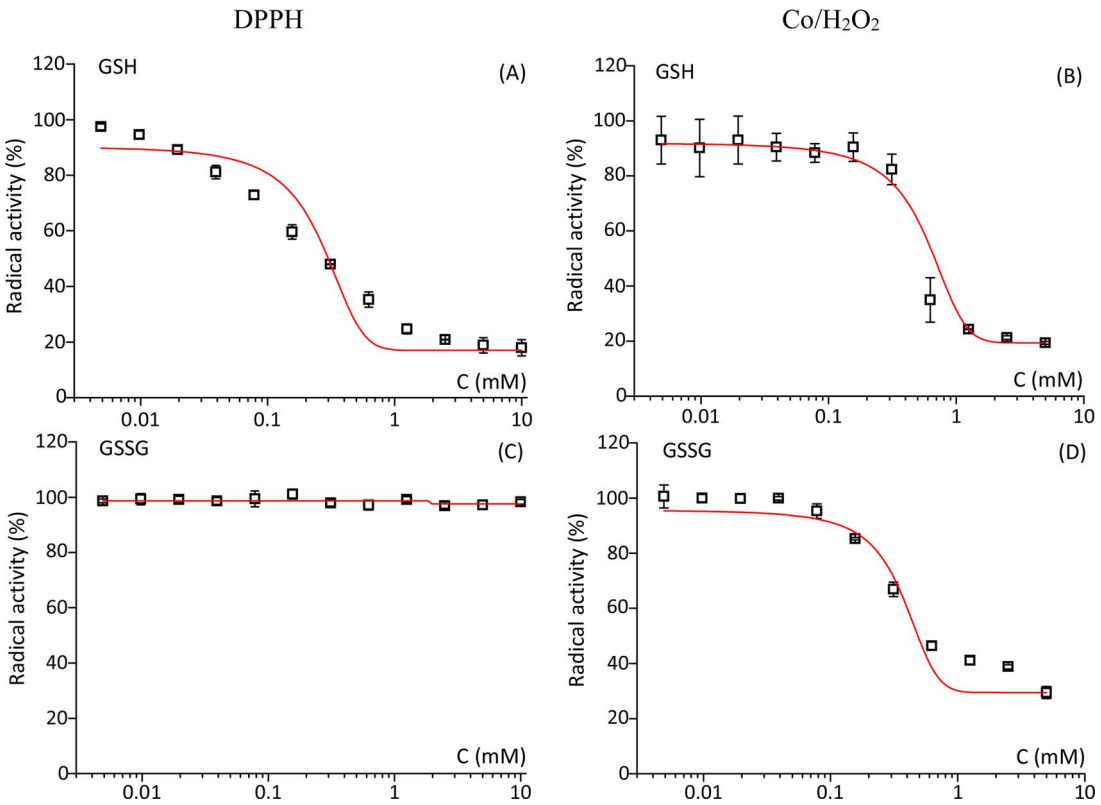


Fig. 3. Concentration–inhibition relationships for reduced glutathione (GSH) and oxidized glutathione (GSSG) according to DPPH and Co/H_2O_2 assays. Mean ± SD (n = 3) are shown.

Table 1
Half-maximal effective concentrations (EC_{50}, μM) of GSH–DTPA salts and the constituents according to DPPH and Co/H_2O_2 assays.

Compounds	DPPH	Co/H_2O_2
1 (GSH)	248 ± 18	544 ± 39
1 (GSSG)	Inactive	365 ± 67
2a (Et*)	215 ± 17	416 ± 7
2b (Bu*)	78 ± 3	924 ± 46
2c (*i*-Pr*)	NA	NA
2d (Menthyl*)	150 ± 20	> 1000
2e (IPC*)	~1000	> 1000
3a	30 ± 3	91 ± 7
3b	18 ± 5	414 ± 63
3c	31 ± 2	261 ± 32
3d	186 ± 4	> 1000
3e	41 ± 7	826 ± 10

*O-organyl substituents of DTPA are shown.

and the corresponding GSH dithiophosphates **3a–3e** were further analyzed and compared. In the case of DPPH assay, EC_{50} values of the DTPA **2a, 2b, 2d** varied from 78 to 215 μM, which were therefore lower than that of GSH (248 μM), whereas the compound **2e** had a relatively low DPPH-scavenging capacity (EC_{50} ≈ 1 mM) (Table 1). These data show that the DTPA possess direct antiradical activity which depends on structure of the O-organyl substituents.

The GSH–DTPA salts **3a–3e** exhibited a noticeably higher capacity for scavenging of the DPPH radical (EC_{50} = 18–186 μM) (Table 1) in comparison with the constituents. In the Co/H_2O_2 assay, most GSH–DTPA salts and DTPA demonstrated a relatively low inhibitory effect, which was comparable or somewhat lower to that of GSH and GSSG. The exception was the O-ethyl substituted GSH–DTPA salt (**3a**) which had EC_{50} value of 91 μM in the latter assay (Table 1). This could be explained by increased reactivity of the GSH moiety in the Fenton reaction when coupled with the O-ethyl substituted DTPA rather than the other DTPAs with extended alkyl and monoterpenyl substituents.

3.2.3. Effect of GSH and GSH–DTPA salts on oxidative burst in vitro

Antioxidant effect of the compounds was evaluated on PC-3 cancer cells, which were sequentially stained with DCFDA and treated with H_2O_2 to induce an oxidative burst. Exposure of the cells to GSH or GSH–DTPA salts was accompanied by different changes in intracellular fluorescence of the probe as a measure of oxidation level (Fig. 4). At an extracellular concentration of GSH as high as 10 mM, which is comparable to or somewhat above its physiological level in cells (1–11 mM [3]), the tripeptide completely suppressed oxidative burst in PC-3 cells to an extent below the background level (Fig. 4). At a lower concentration of 100 μM, the GSH supplement, to the contrary, increased cell fluorescence indicating additional generation of the oxygen radicals, presumably due to alteration of the redox balance in the cells treated [23]. This prooxidant effect disappeared when GSH was used at a concentration of 10 μM (Fig. 4).

The GSH–DTPA salts were applied to PC-3 cells at a concentration of 100 μM in view of their restricted aqueous solubility. The salts containing O-alkyl substituents (**3a, 3b, 3c**) promoted oxidation of the cells by H_2O_2 similarly to that of 100 μM GSH but in a higher extent. To the contrary, the salts containing O-monoterpenyl substituents (**3d, 3e**) significantly decreased intracellular level of the oxygen radicals (Fig. 4) presumably due to both their enhanced antioxidant activity and cellular availability.

3.3. Effect of GSH and GSH–DTPA salts on cell viability

3.3.1. Proliferation of mammalian cells exposed to the compounds

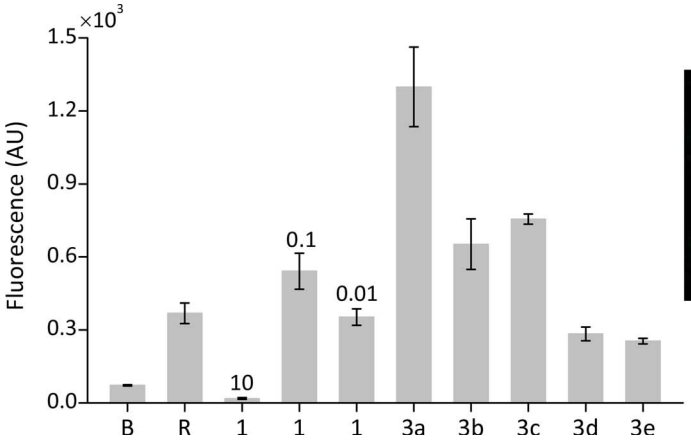
The effect of the GSH–DTPA salts and GSH was studied by using the MTT assay on human skin fibroblasts (HSF) and cancer cell lines including PC-3, MCF-7 and vinblastine-resistant MCF-7 (MCF-7/Vinb) cells. GSH was found to considerably stimulate cell proliferation. In the case of HSF, the stimulating effect appeared at the lower concentration range (< 0.2 μM) and approached ~40% (Fig. 1S, Supplementary data). Proliferation of the cancer cells was promoted by the GSH supplement in a wider concentration range, and this effect increased as follows: MCF-7 (up to 20%), PC-3 (up to 40%), MCF-7/Vinb (~60–110%) (Fig. 1S), presumably, in accordance with a cancerous phenotype of the cells. Hence, a single administration of GSH greatly increases proliferative potential of these cells up two times as observed for the vinblastine-resistant MCF-7 cells.

Unlike GSH, the DTPA and GSH–DTPA salts inhibited proliferation of the cells in a concentration-dependent manner. Corresponding half maximal inhibitory concentrations (IC_{50}) of the compounds are summarized in Table 2. These results show that the DTPAs exhibit moderate cytotoxicity with comparable IC_{50} values mainly within 100–300 μM concentration range. The compound **2b** containing O-butyl substituent had a relatively low effect on viability of HSF (IC_{50} ~1.2 mM).

The GSH–DTPA salts **3a, 3b, 3c** similarly influenced cell viability to that of corresponding O-alkyl substituted DTPA **2a, 2b, 2c** (Table 2), presumably due to a relatively low cellular availability of the former salts. Unlike them, the compounds **3d** and **3e** derived from O-monoterpenyl substituted DTPA decreased cell viability to a much higher extent which was attributed to their enhanced intracellular penetration. Specifically, the compound **3e** based on IPC had similar IC_{50} values for all cell types (18–37 μM), whereas the O-menthyl based compound **3d** exhibited somewhat differentiated effect with IC_{50} values of 4–5 μM for cancer cell lines (PC-3, MCF-7), 1 μM for the vinblastine-resistant MCF-7 cells and 23 μM for HSF.

3.3.2. Effect on mitochondrial potential and membrane lipid peroxidation

Flow cytometric study of the GSH–DTPA salts was carried out using



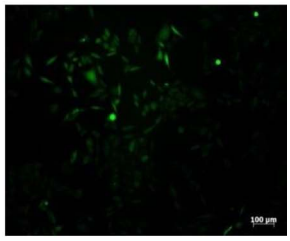


Fig. 4. Effect of GSH and GSH–DTPA salts on intracellular fluorescence of DCFDA probe in H_2O_2-treated PC-3 cells. B – background fluorescence (untreated cells), R – reference (H_2O_2-treated cells), 1–GSH (concentration is shown above columns, mM). The cells were treated with compounds **3a–3e** (0.1 mM) for 60 min. Inset: fluorescent microphotograph of PC-3 cells subjected to oxidative burst. Mean ± SD (n = 3) are shown. Values are significant relative to the reference ($p < 0.05$).

Table 2
Half maximal inhibitory concentrations (IC_{50}, μM) of GSH–DTPA salts in proliferation of mammalian cells (MTT assay, mean ± SD, n = 3).

Compound	PC − 3	MCF-7	MCF-7/Vinb	HSF
2a	193 ± 27	366 ± 10	218 ± 67	193 ± 78
2b	172 ± 24	245 ± 36	153 ± 60	1180 ± 24
2d	108 ± 18	242 ± 57	146 ± 16	158 ± 30
2e	66 ± 5	165 ± 18	115 ± 20	278 ± 64
3a	72 ± 12	350 ± 25	508 ± 40	793 ± 60
3b	94 ± 8	145 ± 16	112 ± 37	322 ± 53
3c	230 ± 34	167 ± 35	245 ± 61	274 ± 53
3d	4 ± 1	5 ± 1	1 ± 0.3	23 ± 2
3e	18 ± 1	30 ± 1	26 ± 4	37 ± 2

fluorescent probes for the transmembrane potential of mitochondria (TMRE) and peroxidation of the plasma membrane lipids (BODIPY 581/591 C11). PC-3 cells were cultured in the presence of **3a–3e** at their IC_{50} concentrations for 48 h followed by staining and analysis of the cells as detailed in the 'Material and methods' section. GSH was applied to the cells for comparison at increased concentration as high as 1 mM due to the lack of its cytotoxic effect.

Fig. 2S (Supplementary data) shows flow cytometric data for the TMRE probe, a delocalized lipophilic cation which accumulates in mitochondria in proportion to the transmembrane potential [24]. The mitochondrial potential was insignificantly affected by both GSH and the GSH–DTPA salts. The lack of effect on the mitochondrial potential under experimental conditions suggests no selective accumulation of the GSH–DTPA salts in mitochondria or their ability to affect mitochondrial functioning.

In addition, the BODIPY 581/591 C11 membrane probe was used, which fluorescence is reduced upon interaction with lipid peroxyl radicals [25]. The mean channel fluorescence of the probe decreased by a factor of 1.1 when the cells were exposed to GSH (Fig. 5, **1**), indicating slight increase in intracellular level of the oxygen radicals in accordance with moderate prooxidant action of GSH on H_2O_2-treated PC-3 cells at submillimolar concentration (Fig. 4).

At IC_{50} concentrations, the GSH–DTPA salts **3a**, **3b**, **3c** showed the lack of effect on cellular fluorescence of the BODIPY 581/591 C11 probe or insignificantly decreased the signal in the case of **3b** (Fig. 5). In contrast to the above compounds, the O-monoterpenyl substituted salts **3d** and **3e** increased the signal by a factor of almost 1.22 (**3d**) and 1.11 (**3e**) (Fig. 5). These data further support that the compounds **3d** and **3e** are more effective cellular antioxidants, which are capable of inhibiting endogenous oxygen radicals causing lipid peroxidation, e.g. hydroxyl radical and hydroperoxyl [26].

4. Discussion

Different O,O-diorganyl DTPA containing alkyl, monoterpenyl, phenyl, and carbohydrate substituents were previously synthesized [15–18,20]. These DTPA were found to form ammonium dithiophosphates with natural amino acids. The coupling reaction is based on quaternization of the most basic nitrogen atom of amino acids by the acidic SH group of DTPA to produce the corresponding ammonium dithiophosphates. In our study, this approach was for the first time extended to oligopeptides with a focus on GSH, a key antioxidant peptide. A series of the GSH dithiophosphates were synthesized by coupling with O-alkyl or O-monoterpenyl substituted DTPA (Fig. 1) as potentially bioactive peptide derivatives.

The GSH–DTPA salts **3a–3e** synthesized are semi-solid in a wide range of temperature and initially insoluble in water. These properties suggest that GSH and DTPA form ionic pairs which are similar to the phosphorylated ionic liquids [27–29]. A growing number of applications of ionic liquids in chemical and pharmaceutical technologies determines the need for investigation of their safety and bioactivity [29,30]. Antioxidant activity of the ionic liquids based on biomolecules, such as peptides, to the best of our knowledge has not been evaluated to date.

A comparative study of antioxidant properties of the GSH–DTPA salts and the constituents was performed using two relevant microplate methods, namely, the colorimetric DPPH assay and fluorometric assay which utilizes Fenton-like reaction (Co/H_2O_2). The DPPH assay has

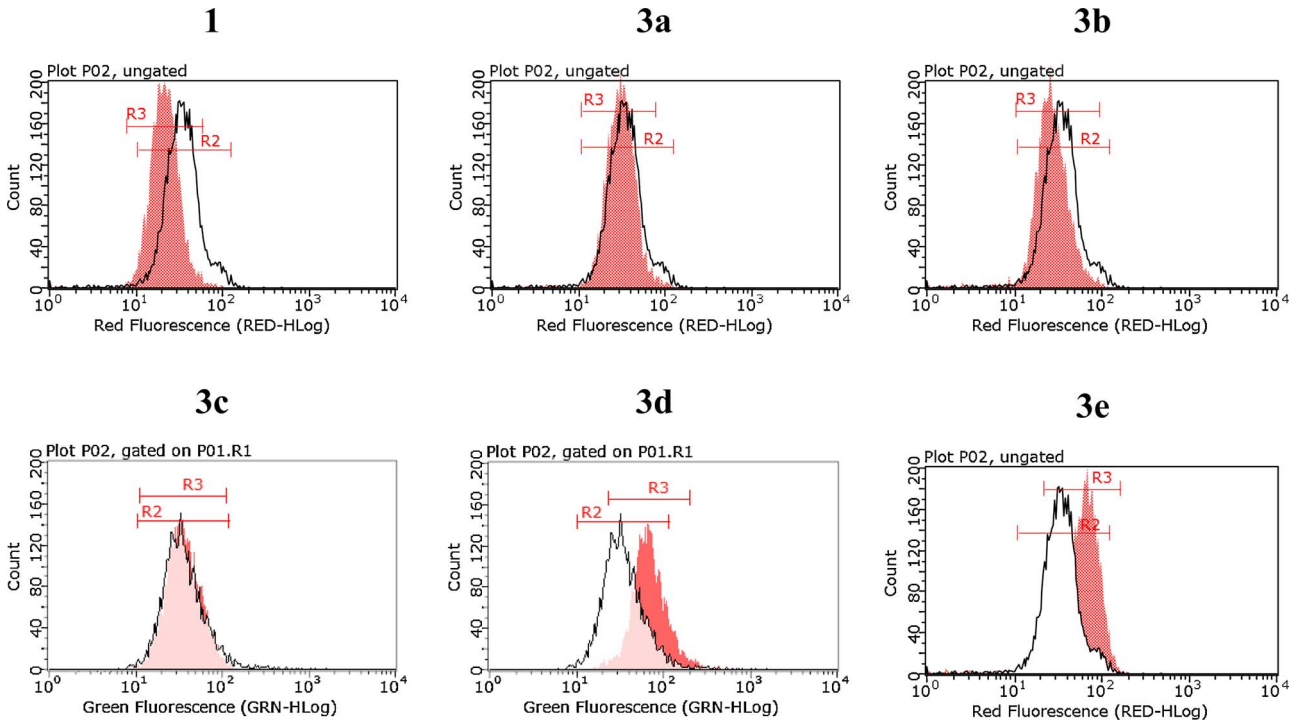


Fig. 5. Fluorescence distribution of 250 nM BODIPY 581/591 C11 in PC-3 cells cultured with GSH (1 mM) and GSH–DTPA salts (IC_{50}) according to flow cytometry. Unfilled curve – untreated cells (reference), filled curve – cells treated with compounds (48 h).

become a routine technique to evaluate radical-scavenging activity of phenolic and lipophilic antioxidants in the reaction with chromogenic DPPH radical, mainly, in alcohol solutions [31,32]. To assess hydrophilic peptides in aqueous solutions, a detergent-assisted protocol with increased DPPH solubility was proposed [21]. Along with the DPPH assay, the other methods targeted at physiologically relevant prooxidant reactions, such as Fenton reactions, are of particular interest in studying antioxidant peptides. The methods for detection of Fe/H_2O_2 and Co/H_2O_2 reactions using, respectively, 1,10-phenanthroline [33] and fluorescein [34] as quenching probes were proposed.

In our study, the above approach was further developed and improved to provide a robust and sensitive assay for Fenton reactions by detecting a gradual increase of fluorescence of DCFDA as a sensitive probe for the oxygen radicals, such as hydroxyl radical [35] (Fig. 2). Under experimental conditions the ferrous ions, the most abundant Fenton metal ions, exhibit the lack of prooxidant activity as a result of precipitation during the reaction. The Co/H_2O_2 reaction, to the contrary, is characterized by the highest capability of generating oxygen radicals along with the reproducible signal which linearly depends on time for at least 60 min (Fig. 2). Such activity of cobalt ions apparently reflects their known prooxidant effects on mammalian cells, such as induction of hypoxia-like state and activation of oxygen radical-dependent transcriptional factors [36,37]. Hence, the Co/H_2O_2 Fenton-like system was used to establish the fluorometric assay for antioxidant peptides.

The use of the above assays together allows for more informative evaluation of antioxidant properties of GSH and its derivatives. The DPPH assay is primarily sensitive to the thiol group of GSH, whereas generation of the oxygen radicals in the Co/H_2O_2 assay may be affected by different groups of the tripeptide. GSH and its derivatives can act as ligands for different metals, including Fenton ones, with several sites available [38–41]. γ-Glutamyl residue of GSH is a probable site for binding of cobalt ions [38]. The complex formation between the tripeptide and Co apparently results in partial inhibition of the Fenton reaction, in which GSSG produces even higher inhibitory effect than GSH when the tripeptide is used in deficiency relative to Co (Fig. 3B and D). At higher concentrations (GSH/Co ratio), GSH provides more complete suppression of the reaction by ~80% compared with GSSG due to participation of the thiol groups (Fig. 3B and D).

According to the both assays, the GSH–DTPA salts possess enhanced radical-scavenging activity compared with the constituents (Table 1). This effect is not achieved when the compositions of GSH and DTPA are used, supporting the appearance of new antioxidant properties in the GSH dithiophosphates. Our data also suggest that the O-diorganyl substituents modulate reactivity of the DTPA and GSH–DTPA salts in the oxidative reactions (Table 1) by providing different surrounding for active sites of the compounds, such as probably metal-binding γ-glutamyl residues in GSH (Fig. 1).

The effect of GSH and its dithiophosphates on oxidation level in mammalian cells was studied on PC-3 cell monolayers treated with H_2O_2 (Fig. 4). The results demonstrate a dose-dependent effect of extracellularly applied GSH which varies from strong antioxidant to moderate prooxidant one at millimolar and submillimolar concentrations, respectively. This dual activity of GSH is in accordance with the existing data on inherent prooxidant properties of natural antioxidants including GSH [23,42,43] and ascorbic acid [44,45]. In particular, GSH as well as its precursor NAC were reported to exhibit prooxidant effects depending on nature of the free radical and reaction conditions [42]. One of the probable mechanisms of this effect results from the ability of thiols to reduce metal ions, thus regenerating them for redox reactions [23].

According to the in vitro assay, the GSH–DTPA salts (0.1 mM) applied to the H_2O_2-treated cells are characterized by compound-specific pro- or antioxidant activity (Fig. 4). This opposite effect of the compounds could be attributed to their different ability to pass across the plasma membrane of cells, which in turn should affect their intracellular concentration and manifestation as a pro- or antioxidant. In view of this assumption, the O-menthyl substituent in **3d** and O-isopinocampheolyl substituent in **3e** provide more effective cellular accumulation of the compounds than the O-alkyl substituents in **3a, 3b, 3c**. This is supported by membrane-modulating and penetration-enhancing properties of monoterpenols [46,47] as well as increased antiproliferative (cytotoxic) activity of the compounds (Table 2).

In particular, the GSH–DTPA salts **3d** and **3e** are generally up to ~2 orders of magnitude more cytotoxic (IC_{50} = 1–37 μM) compared with **3a, 3b, 3c** (Table 2), further supporting increased cellular availability of the former compounds. Interestingly, the synthesized GSH–DTPA salts **3a–3e** exhibit substantially lower cytotoxic concentrations than the ionic liquids composed of different ionic pairs (IC_{50} values reported for different cancer cell lines are generally in the millimolar concentration range) [29,48–50]. In particular, IC_{50} values of the ionic liquids based of choline and different O,O-dialkyl phosphate, phosphinate and dithiophosphates for J774 murine macrophage cells vary from 0.25 to 20.4 mM [29]. It should be noted however that these cytotoxicity studies [29,48–50] were performed with modifications, e.g. at a higher density of the seeded cells and less exposure time (24–48 h), which should result in some underestimation of the cytotoxic effect. Nevertheless, our data demonstrate the enhanced effect of the GSH–DTPA salts on viability of mammalian cells suggesting a potentially high bioactivity of these compounds.

As shown in Fig. 1S, the unmodified GSH in contrast to its dithiophosphates substantially increases cell proliferation in vitro. A decrease in the intracellular GSSG/GSH ratio due to increase of GSH pool seems to be responsible for this effect as reported for extracellularly applied thiols [51,52]. The GSSG/GSH ratio is a key parameter of the redox homeostasis which controls a balance between the proliferation, differentiation and apoptosis of cells [2].

Both the GSH–DTPA salts and GSH differently influence viability of the cancer cells in comparison with the early-passaged HSFs that maintain characteristics of untransformed 'normal' cells. Specifically, both stimulating effect of GSH and inhibitory ability of the GSH–DTPA salts on cell proliferation is more profound for the cancer cells, e.g. MCF-7/Vinb and PC-3 than for HSF (Fig. 1S, Table 2). Such selectivity could be considered from a view of altered metabolism of the cancer cells which features aerobic glycolysis, mitochondrial abnormalities, and elevated levels of oxygen radicals (prooxidants) to support high proliferative potential [53,54]. These metabolic features should result in increased sensitivity of the cancer cells to redox modulating agents, which may both suppress or promote the oxidative metabolism.

The supplementation of GSH at relatively low concentrations is expected to alleviate the oxidation processes and promote propagation of the cancer cells (Fig. 1S). This observation supports the risk of inappropriate administration of antioxidants in promoting tumors growth and increasing their survival upon treatment with oxygen radical-generating anticancer drugs [55]. The GSH–DTPA salts seem to disturb redox metabolism in a much more effective way than GSH due to their enhanced antioxidant properties and cellular availability, resulting in increased cytotoxicity compared with the constituents. The fact that the most active GSH dithiophosphates, namely based on the monoterpenyl substituted DTPA (**3d** and **3e**) have higher cytotoxicity for the cancer cells than HSF (Table 2) further supports the redox modulating ability of the compounds. According to our in vitro data, the GSH dithiophosphates **3d** and **3e** are promising GSH derivatives with enhanced antioxidant and antiproliferative properties. Other potential activities of the GSH–DTPA salts, such as inhibitory activity towards GSH-dependent enzymes, will be studied elsewhere.

5. Conclusions

Our results show that the O,O-diorganyl substituted DTPA are promising modifying agents for GSH, which substantially modulate its antioxidant properties and cellular availability when coupled with the

tripeptide through ionic interactions. The coupling reaction involves quarternization of the amino group of γ-glutamyl residue in GSH and results in the stable GSH dithiophosphates. Such GSH–DTPA salts possess enhanced redox modulating activity in vitro compared with the constituents. Our results demonstrate that the O-monoterpenyl substituted DTPA generate more active GSH dithiophosphates than the O-alkyl substituted ones. In view of diverse effects of the monoterpenes including carrier, antioxidant and anti-inflammatory activities [56,57], the O-monoterpenyl substituted GSH dithiophosphates can be considered as candidates for bioactive compounds. Owing to their redox modulating and antiproliferative properties, these compounds have a potential in antitumor, antimicrobial and antiviral therapy. The chemical approach proposed can be extended to various natural and synthetic oligopeptides.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.peptides.2017.10.002.

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