Amino Acids (2012) 42:261–269
DOI 10.1007/s00726-010-0804-z

ORIGINAL ARTICLE

CNS delivery of L-dopa by a new hybrid glutathione–methionine peptidomimetic prodrug

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Received: 20 August 2010 / Accepted: 29 October 2010 / Published online: 17 November 2010

Abstract Parkinson's disease (PD) is a neurodegenerative disorder associated primarily with loss of dopamine (DA) neurons in the nigrostriatal system. With the aim of increasing the bioavailability of L-dopa (LD) after oral administration and of overcoming the pro-oxidant effect associated with LD therapy, we designed a peptidomimetic LD prodrug (**1**) able to release the active agent by enzyme catalyzed hydrolysis. The physicochemical properties, as well as the chemical and enzymatic stabilities of the new compound, were evaluated in order to check both its stability in aqueous medium and its sensitivity towards enzymatic cleavage, providing the parent LD drug, in rat and human plasma. The radical scavenging activities of prodrug **1** was tested by using both the DPPH–HPLC and the DMSO competition methods. The results indicate that the replacement of cysteine GSH portion by methionine confers resistance to oxidative degradation in gastric fluid. Prodrug **1** demonstrated to induce sustained delivery of DA in rat striatal tissue with respect to equimolar LD dosages. These results are of significance for prospective therapeutic application of prodrug **1** in pathological events associated with free radical damage and decreasing DA concentration in the brain.

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Keywords L-Dopa · Glutathione · Methionine · Parkinson's disease

Introduction

Parkinson's disease (PD) and Alzheimer's disease (AD) are progressive neurodegenerative disorders that affect an increasing number of the elderly population. Although current drug therapy of PD turned out to be more successful compared to that of AD, it does not stop the degenerative process, and pharmacological treatments lose effectiveness with progression of the disease (Everts et al. 2001). Dopamine (DA) shortage is mainly responsible for the motor deficits of the disorder and several drugs, that boost the levels of DA or mimic its effects, are available for treating PD; nevertheless, none of them exceeded the clinical efficacy of their biological precursor L-dopa (LD) (LeWitt 2008). Since its introduction in the late 1960s, LD, which counteracts parkinsonian motor symptoms by restoring the nigrostriatal DA deficiency, still remains the key compound of pharmacotherapy for PD to which all other therapies are compared. Up to now, several compounds have been studied with the aim of enhancing LD chemical stability and water or lipid solubility, and diminishing its susceptibility to enzymatic degradation. During the last two decades the prodrug approach was demonstrated to be quite promising in addressing the problem of the rapid in vivo degradation of LD, and consequently, in improving its bioavailability (Wang et al. 1995; Marrel et al. 1985; Cooper et al. 1984; Garzon-Aburbeh et al. 1986; Cooper et al. 1987; Ihara et al. 1989; Cingolani et al. 2000; Giorgioni et al. 2010). In recent years an ameliorative version of the prodrug approach, developed for favoring the delivery of LD to the central

nervous system (CNS), has involved the development of dual-acting prodrugs. A few papers reported on the efficacy of LD, covalently linked to different antioxidant molecules, to induce sustained release of drug in rat striatum and, at the same time, to protect against the oxidative stress deriving from autoxidation of DA (More and Vince 2008; Sozio et al. 2008; Di Stefano et al. 2006; Pinnen et al. 2007; Pinnen et al. 2009). In order to efficiently deliver small, hydrophilic molecules across the BBB two different strategies are currently used: (1) the chemical modification of hydrophilic molecules to more lipophilic derivatives, named lipidization; and (2) the transporter-mediated delivery, in which the substrate of one of the numerous transporters located within the BBB is exploited as the carrier moiety of the drug (Pardridge 2009).

Several studies showed that the transport of many peptides can be accounted for on the basis of their size and lipophilicity and a number of peptide transport systems have been described in the BBB. In this respect, several di- and tri-peptide mimetic prodrugs of DA and LD have been shown to increase the oral bioavailability of LD together with improved BBB permeability (Bai 1995; Malakoutikhah et al. 2008; Wang et al. 1995; More and Vince 2008). On the basis of these findings and in an attempt to increase the BBB penetration of LD, we developed a novel hybrid glutathione–methionine peptidomimetic operating as a dual-acting LD prodrug. This hybrid peptidomimetic is constituted by the following components: the carrier, a modified GSH in which cysteine is replaced with methionine, the drug, an acetylated LD methylester, and the linker, an amide bond which connect LD with the glycine residue of the modified GSH (Fig. 1). The rationale behind the design of this new compound is based on previous studies on glutathione peptidomimetics as components of antiParkinson prodrugs performed by More and Vince (2008) and Pinnen et al. (2007) and on the observation of Malakoutikhah et al. (2008). The former demonstrated an interaction between the GSH residues of the investigated prodrug and the GSH

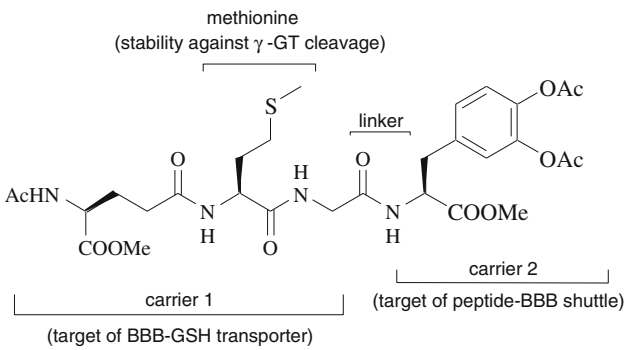


Fig. 1 Chemical structure of multifunctional prodrug **1**

transporters located on the luminal side of the BBB. The latter demonstrated the capacity of different peptides to transfer levodopa through an artificial membrane by means of passive diffusion via peptide BBB-shuttles. The replacement of cysteine by methionine in GSH has been shown to favor its antioxidant activity at pH 4 rather than at pH 1, as is the case of native GSH, thus conferring resistance to oxidative degradation in the gastric fluid (Bobrowski et al. 2007). The release of radical scavenging molecules such as the here investigated glutathione peptidomimetic residue inside the CNS would most likely contribute in attenuating the damage caused by the oxidative stress commonly associated with PD and by the pro-oxidant effects of traditional LD therapies. Furthermore, the incorporation of methionine in the GSH residue would add to the prodrug the benefit of conferring stability against γ-glutamyl transpeptidase (γ-GT) cleavage. In order to propose the new compound **1** as a prospective prodrug for PD, we have investigated in the present study its biological and physicochemical properties as well as the rates of chemical and enzymatic hydrolysis.

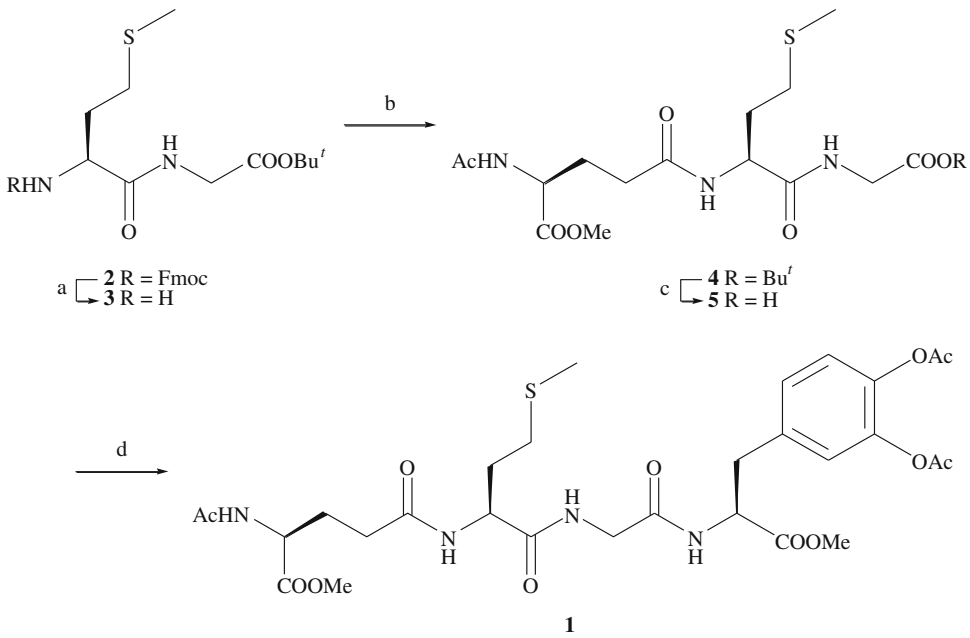
Chemistry

Fmoc-Met-Gly-OBu^t (**2**) and H-LD(Ac)$_2$-OMe·HCl were synthesized as previously reported (Stöber et al. 1997; Bodor et al. 1977).

Prodrug **1** was synthesized as outlined in Scheme 1 by employing solution phase procedures through elongation of the protected peptide chain **5** in the C-direction with the LD derivative.

Experimental section

Microanalyses were performed on a 1106 Carlo Erba CHN analyzer. ^{1}H- and ^{13}C-NMR spectra were recorded on a Varian VXR 300-MHz spectrometer. Chemical shifts are reported in parts per million (δ) downfield from the internal standard tetramethylsilane (Me_4Si). The LC–MS/MS system consisted of an LCQ (Thermo Finnigan) ion trap mass spectrometer (San Jose, CA, USA) equipped with an electrospray ionization (ESI) source. The capillary temperature was set at 300°C and the spray voltage at 4.25 kV. The fluid was nebulized using nitrogen (N_2) as both the sheath gas and the auxiliary gas. The identity of the new prodrug was confirmed by elemental analysis, NMR data and LC–MS/MS measurements; homogeneity was confirmed by TLC on silica gel Merck 60 F_{254}. Solutions were routinely dried over anhydrous sodium sulphate prior to evaporation. Chromatographic purifications were performed by Merck 60 70–230 mesh ASTM silica gel column.



Scheme 1 Reagents and conditions. **a** DBU, DCM, DCC, 20 min, rt; **b** Ac-Glu-OMe, HOBt, DCC, DMF, 3 h, 0°C then 15 h, 4°C; **c** TFA, 2 h, rt; **d** H-LD(Ac)$_2$-OMe·HCl, IBCF, TEA, DMF, 3 h, 0°C then 15 h, 4°C

Chemicals

Ac-Glu-OMe was obtained from Bachem. All other chemicals were of the highest commercially available purity.

H-Met-Gly-OBu^t (3) To a solution of the N-protected dipeptide *t*-butyl ester **2** (5 g, 10.3 mmol) in CH_2Cl_2 (70 mL) DBU (1.57 g, 10.3 mmol) was added at room temperature. After 20 min the solution was evaporated to dryness and the residue chromatographed on silica gel using $CHCl_3$:MeOH (95:5) as eluant to yield pure N-deprotected dipeptide *t*-butyl ester **3** (1.68 g, 62%). $R_f = 0.43$, $CHCl_3$:MeOH (95:5); ^{1}H-NMR ($CDCl_3$) δ: 1.44 (9H, s, OBu^t), 2.45 (2H, s br, NH_2), 1.65–1.80 (1H, m, Met β-CH_A), 2.15 (3H, s, Met CH_3), 2.16–2.20 (1H, m, Met β-C_B), 2.45–2.55 (2H, m, Met γ-CH_2), 3.50 (1H, m, Met α-CH), 3.85–3.90 (2H, m, Gly α-CH), 7.84 (1H, s br, Gly NH). ^{13}C-NMR ($CDCl_3$) δ: 15.48 (Met CH_3), 28.24 (OBu^t), 30.77 (Met β-CH_2), 34.19 (Met γ-CH_2), 41.88 (Gly α-CH), 54.31 (Met α-CH), 82.33 (OBu^t), 169.28 and 175.02 (2 × CO).

Ac-Glu[-Met-Gly-OBu^t]-OMe (4) To a stirred solution of N-deprotected dipeptide *t*-butyl ester **3** (1.5 g, 5.7 mmol) in dry DMF (7 mL) Ac-Glu-OMe (1.16 g, 5.7 mmol) in dry DMF (7 mL) was added at 0°C followed by portion-wise addition of HOBt (0.77 g, 5.7 mmol) and DCC (1,10 g, 5.7 mmol) in dry DMF (8 mL). After 3 h at 0°C and 16 h at 5°C, the reaction mixture was filtered and the resulting solution was evaporated under vacuum. The residue was taken up in EtOAc and the organic layer washed with 1 N $KHSO_4$, saturated aqueous $NaHCO_3$ and brine. The residue obtained after drying and evaporation was chromatographed on silica gel by using $CHCl_3$:MeOH (95:5) as the eluant to give the corresponding pure tripeptide *t*-butyl ester **4** (1.97 g, 77%). $R_f = 0.30$, $CHCl_3$:MeOH (95:5); ^{1}H-NMR ($CDCl_3$) δ: 1.44 (9H, s, OBu^t), 1.96–2.01 (2H, m, Met β-CH_2), 2.02 (3H, s, Met CH_3), 2.11 (3H, s, Glu Ac), 2.16–2.20 (2H, m, Glu β-CH_2), 2.33-2.38 (2H, m, Glu γ-CH_2), 2.55–2.63 (2H, m, Met γ-CH_2), 3.74 (3H, s, OMe), 3.91–3.93 (2H, m, Gly α-CH), 4.56 (1H, m, Glu α-CH), 4.65 (1H, m, Met α-CH), 6.65 (1H, d, $J = 7.2$ Hz, Glu NH), 6.89 (2H, m br, Mat and Gly NH); ^{13}C-NMR ($CDCl_3$) δ: 15.41 (Met CH_3), 23.25 (Glu Ac), 28.25 (OBu^t), 29.89 (Glu β-CH_2), 30.36 (Met β-CH_2), 31.38 (Glu γ-CH_2), 32.45 (Met γ-CH_2), 42.21 (Gly α-CH), 52.02 (OCH_3), 52.33 (Glu α-CH), 52.77 (Met α-CH), 82.57 (OBu^t), 168.92, 170.81, 171.47, 172.53 and 172.75 (5 × CO).

Ac-Glu-[Met-Gly-LD(Ac)$_2$-OMe]-OMe (1) The above reported tripeptide *t*-butyl ester **4** (1.80 g, 4.02 mmol) was dissolved in TFA (5.1 mL). After 2 h at room temperature the solution was evaporated to dryness and the residue repeatedly evaporated with ether to give **5** in quantitative yield. This product was used without further purification (1.54 g, 98%).

To an ice-cold solution of deprotected tripeptide **5** (1.40 g, 3.58 mmol) in dry DMF (5 mL) TEA (0.50 mL, 3.58 mmol) and isobutyl chloroformate (IBCF) (0.47 mL, 3.58 mmol) were added under stirring. After 15 min at −15°C, H-LD(Ac)$_2$-OMe·HCl (1.19 g, 3.58 mmol) in TEA (0.50 mL, 3.58 mmol) and dry DMF (5 mL) were added to the mixture at −15°C with stirring. After 3 h at 0°C and 16 h at 5°C, the reaction mixture was filtered and the

resulting solution was evaporated under vacuum. The residue was taken up in AcOEt and the organic layer washed with 1 N $KHSO_4$, saturated aqueous $NaHCO_3$ and brine. The residue obtained after drying and evaporation was crystallized from AcOEt (0.96 g, 40%). $R_f = 0.41$, $CHCl_3$:MeOH (95:5); ^{1}H-NMR (d_6-DMSO) δ: 1.77–1.94 (4H, m, Glu and Met β-CH_2), 1.90 (3H, s, Met CH_3), 2.01 (3H, s, Glu Ac), 2.09–2.55 (4H, m, Glu and Met γ-CH_2), 2.23 and 2.25 (6H, 2 × s, LD Ac), 2.95–3.10 (2H, m, LD β-CH_2), 3.65 (3H, s, OMe), 3.70 (3H, s, OMe), 3.72–3.80 (2H, m, Gly α-CH_2), 4.10–4.25 (2H, m, Glu and Met α-CH), 4.45 (1H, m, LD α-CH), 7.10–7.20 (3H, m, Ar), 8.05 (1H, d, $J = 7.2$ Hz, Glu NH), 8.10 (1H, t, $J = 5.8$ Hz, Gly NH), 8.15 (1H, d, $J = 6.89$ Hz, Met NH), 8.25 (1H, d, $J = 7.1$ Hz, LD NH); ^{13}C-NMR ((d_6-DMSO) δ: 15.21 (Met CH_3), 21.03 and 21.05 (LD 2 × Ac), 22.94 (Glu Ac), 27.36 (Glu β-CH_2), 30.21 (Met β-CH_2), 31.90 (Glu γ-CH_2), 32.17 (Met γ-CH_2), 36.53 (LD β-CH_2), 42.19 (Gly α-CH), 52.14 (OCH_3), 52.47 (Glu α-CH), 52.57 (OCH_3), 52.76 (Met α-CH), 54.09 (LD α-CH), 123.97, 124.83, 127.89, 136.50, 141.34 and 142.31 (Ar), 168.87, 168.92, 169.57, 170.13, 172.23, 172.32 and 173.22 (6 × CO). Anal. calculated for ($C_{29}H_{40}N_4O_{12}S$): C, 52.09; H, 6.03; N, 8.38; S, 4.80; found: C, 52.29; H, 6.02; N, 8.40; S, 4.65. MS (ESI) *m/z* 669 $(M–H)^-$.

Chromatographic conditions

Analytical HPLC measurements were run on a Waters 1525 Binary HPLC pump, equipped with a Waters 2996 photodiode array detector, a 20-µL Rheodyne injector and a computer integrating apparatus. The column was a Waters Symmetry RP-C_{18} column (4.6 × 150 mm, 5 µm), the mobile phase was a mixture of water/methanol (10:90) at a flow rate of 1 mL/min, the UV-detector was set at a length of 264 nm.

Pharmacokinetic analysis were run on the HPLC system consisted of a Waters 600 controller pump, a Rheodyne 7295 injector with a 10-µL loop and an Antec Leyden Decade II detector; the operating potential was 0.75 V. Separation was achieved on a Waters Symmetry RP-C_{18} column (4.6 × 150 mm, 5 µm). The mobile phase consisted of 0.045 M monobasic sodium phosphate, 0.001 M 1-octanesulphonic acid sodium salt, 0.006% triethylamine, 0.015% 100 µM sodium EDTA and 6% acetonitrile. The pH of the mobile phase was adjusted to 3.0 by *o*-phosphoric acid. The mobile phase was filtered and degassed by vacuum. A flow rate of 1 mL/min was used in all experiments. Monoamine stock solutions were prepared at a concentration of 1 mg/mL (as a free base) in 0.05 N perchloric acid containing 0.064% 1-octanesulphonic acid sodium salt, 0.060% heptanesulphonic acid sodium salt, 0.004% sodium EDTA, and 0.010% sodium metabisulphite. These standard solutions were freshly prepared every week and stored at 4°C for use right away. The monoamine and their metabolites were identified on the basis of retention time. Measurements were performed in triplicate for each original sample.

Aqueous solubility

Compound **1** (50 mg) was placed in a microtube containing 1 mL of deionized water or buffer solutions (at pH 7.4, 5.0 and 1.3 by using 0.02 M phosphate buffer, 0.02 M acetate buffer or 0.02 M hydrochloridic acid buffer, respectively) and shaken at 25°C for 1 h to ensure the solubility equilibrium. After centrifugation, a 20-µL portion of the supernatant was analyzed by HPLC (Pinnen et al. 2009).

Lipophilicity

clog P

The calculated clog *P* was determined by using ACD Log *P* software package, version 4.55 (Advanced Chemistry Development Inc., Toronto, Canada).

Octanol/water partition coefficient

Octanol/water partition coefficient (log *P*) was determined by placing approximately 5 mg of compound **1** in 1 mL of aqueous saturated *n*-octanol, shaking vigorously for about 2 min and filtering. An equal volume of phosphate buffer pH 7.4 was added and the mixture was equilibrated by repeated inversions of up to 200 times for 5 min and then allowed to stand for 30 min for the phases to fully separate. Thereafter the respective phases were analyzed by HPLC.

Log k_0

Solute hydrophobicity of different compounds can be estimated from the corresponding retention times (RTs) due to the good relationship between log octanol/water partition coefficient and logarithmic retention factors in reverse-phase chromatography (log *k* value) determined by using octadecyl silica columns. (Vailaya and Horváth 1998; Angelini et al. 2005). Retention in reverse-phase chromatography increases with solute lipophilic character and, for a given combination of solute and stationary phase, with the water content of the mobile phase.

Compound **1** was dissolved in methanol (concentration 1 mg/mL). Aliquots of this solution were filtered and analyzed by HPLC. The mobile phase consisted of acetonitrile and water with acetonitrile content varying in the range 90–55% (v/v) with 5% increments (Bajda et al. 2007).

The dead time was measured by injection of methanol as a non-retained compound. The isocratic capacity factor, log k, was calculated from the dead time (t_0) and the retention time (t_r) values by using the following equation (1):

$$\log k = \log\left[\frac{t_r - t_0}{t_0}\right] \quad (1)$$

By plotting log k values against the acetonitrile content it is possible to calculate, by extrapolation to zero acetonitrile percentage, the log k_0 value, that is the solute retention with pure water as the eluent.

Kinetics of chemical hydrolysis

A 0.02 M hydrochloridic acid buffer of pH 1.3 as non-enzymatic simulated gastric fluid (SGF) and a 0.02 M phosphate buffer of pH 7.4 were used in this study. Reactions were initiated by adding 1 mL of 10^{-4} M stock solution (in acetonitrile) of prodrug **1** to 10 mL of the appropriate thermostatted (37 ± 0.5°C) aqueous buffer solution, containing 20% acetonitrile. At appropriate time intervals, samples of 20 µL were withdrawn and analyzed by HPLC. Pseudo-first-order rate constants (k_{obs}) for the hydrolysis of the prodrug were then calculated from the slopes of the linear plots of log (% residual prodrug) against time. The experiments were run in triplicate and the mean values of the rate constants were calculated.

Kinetics of enzymatic hydrolysis

Plasma from rats and human was obtained by centrifugation of blood samples containing 0.3% citric acid at 3,000×g for 15–20 min. Plasma fractions (4 mL) were diluted with 0.02 M phosphate buffer (pH 7.4) to give a final volume of 5 mL (80% plasma). Incubations were performed at 37 ± 0.5°C using a shaking water bath. The reactions were initiated by adding 100 µL of a stock solution of drug (1 mg/mL in acetonitrile) to 5 mL of preheated plasma. Aliquots (100 µL) were taken at various times and deproteinized by mixing with 200 µL of 0.01 M HCl in methanol. After centrifugation for 5 min at 5,000×g, 10 µL of the layer supernatant was chromatographed as described above. The logarithms of the remaining original prodrug were plotted as a function of incubation time in order to give the corresponding k_{obs}.

Radical scavenging activity

DPPH–HPLC method

The DPPH radical scavenging activity was estimated according to the method previously reported (Sozio et al. 2010). The DPPH peak was monitored at 517 nm and the radical scavenging activity was calculated from the peak area (PA) as reported above:

$$\text{Radical scavenging } (\%) = \left[\frac{PA_{blank} - PA_{sample}}{PA_{blank}}\right] \times 100$$

DMSO competition method

The hydroxyl radical production was determined according to Nash (1953). When the hydroxyl radicals, generated by mixing the Fe^{3+}/ascorbic acid system, react with DMSO, the methyl radical forms and then partly transforms into formaldehyde. The reaction mixture (300 µL) was prepared as previously reported (Melagraki et al. 2009): EDTA (0.1 mM), Fe^{3+} (167 mM), DMSO (33 mM) in phosphate buffer (50 mM, pH 7.4), the tested compounds (concentration 0.1 mM) and ascorbic acid (10 mM). After 30 min of incubation (37°C) the reaction was stopped with 100 µL of CCl_3COOH (17% w/v) and then 400 µL of ethyl 3-oxobutanoate were added. After 20 min at 60°C, the mixture was filtered and the formaldehyde derivative peak was detected by HPLC–UV at 365 nm (Burini and Coli 2004). The competition with DMSO was expressed as percent of inhibition of formaldehyde production and was calculated from peak area (PA) as reported above:

$$\text{inhibition } (\%) = \left[\frac{PA_{blank} - PA_{sample}}{PA_{blank}}\right] \times 100 \quad (2)$$

Animals

Male Wistar rats ($n = 75$) (Harlan, UD, Italy) weighing 250–300 g were employed. Five rats were assigned to each treatment group. The animals were housed in plastic (Makrolon) cages in a temperature-controlled room (21 ± 5°C) and maintained on a laboratory diet and water ad libitum. The light/dark cycle was from 7 a.m. to 7 p.m.

Drug administration

Benserazide hydrochloride, a peripheral dopa-decarboxylase inhibitor, was dissolved in water whereas the prodrug **1**, LD and tripeptide were dissolved in dimethyl sulfoxide. All animals received a dose of benserazide (0.083 mmol/kg) combined with **1**, LD or tripeptide in equimolar doses (0.332 mmol/kg). The drugs were given at a volume of 5 ml/kg in a single oral administration by intragastric tube. This study was carried out in accordance with the Italian government's guidelines for the care and use of laboratory animals (D.L. n. 116 of January 27, 1992).

Pharmacokinetic analysis

After slight anesthesia with carbon monoxide, the blood of rats, was collected, by cardiac puncture, in vials containing heparin (250 IU), centrifuged at 2,000×*g* for 10 min and kept at −80°C until analysis. Aliquots (400 µL) were taken at various times and deproteinized by mixing with 40 µL of 4 M perchloric acid. After centrifugation for 5 min at 5,000×*g* and filtration (Millipore 0.45 µm), 10 µL of the layer supernatant was chromatographed as described below. The amounts of LD were plotted as a function of incubation time.

The striatum tissue of the rats were dissected out, frozen as well in liquid nitrogen and stored at −80°C until use. The sampling schedules were 1, 2, 4 and 6 h after treatment with drugs. All striatal tissues were individually homogenized for 2 min with a Dyna-Mix homogenizer (Fisher Scientific) in 500 µL of 0.05 N perchloric acid solution containing (by weight/volume) 0.064% 1-octanesulphonic acid sodium salt, 0.060% heptanesulphonic acid sodium salt, 0.004% sodium EDTA, 0.010% sodium metabisulfite and 25 ng/mL dihydroxybenzylamine (DHBA) as an internal standard. The whole procedure was carried out on ice. The resulting homogenate was then centrifuged at 4,500 g for 10 min and the supernatant was filtered by using 0.45 µm Millipore filters. For each measurement, 10 µL of the obtained filtrate were injected into the liquid chromatography equipment (Giorgioni et al. 2010).

Statistical analysis

DA concentrations in rat striatal tissue are given as percentages of mean values with respect to basal levels. Basal concentrations were determined as the mean of at least three measurements with ≤5% variation obtained at the beginning of the experiment. Data are expressed as means with error bars for standard deviations of five rats. One-way ANOVA was used to evaluate the effect of procedures on each group of animals. If a general effect was determined by ANOVA, post hoc analysis was performed with the Scheffe or Student's test with $P \leq 0.05$ used as the level of significance.

Results and discussion

The physicochemical properties, water solubility and *n*-octanol/water partition coefficient for prodrug **1** were determined in order to predict its absorption, distribution and brain bioavailability. It is generally accepted that good absorption of an orally administered drug could be obtained when the aqueous solubility is more than 10 µg/mL (Yalkowsky and Morzowich 1980) and therefore, to assess this potential, solubility of the prodrug **1** was determined in water and buffer solutions (at pH 1.3, 5.0 and 7.4). From the data reported in Table 1 it appears that the solubility is relatively high in water (6.69 mg/mL) and it decreases in buffers. As expected, the solubility slightly decreases upon increasing the pH of the buffer, although it remains in all cases much higher than the critical value reported by Madhukar (Madhukar et al. 2010).

In order to evaluate the penetration of a molecule through the BBB, the lipophilicity was estimated by using the apparent partition coefficient (log P_{app}) measured in *n*-octanol/phosphate buffer at pH 7.4 and the log capacity factor (log k_0) calculated from reverse-phase chromatographic retention times with acetonitrile and water as the eluents (Bonina et al. 1996). For the sake of comparison, the lipophilicity of prodrug **1** was also calculated using the ACD Log P software package, version 4.55 (Advanced Chemistry Development Inc., Toronto, Canada). Data reported in Table 1 point out that compound **1** is quite hydrophilic as evidenced by negative log *P* and clog *P* values. The value of log $k_0 = 1.21$ is in agreement with a low retention in reverse-phase chromatography as expected for not very lipophilic molecules (Di Stefano et al. 2008).

Stability studies of the new compound were performed at 37°C in isotonic sodium phosphate buffer (pH 7.4), in simulated gastric fluid (SGF, pH 1.3) and in rat and human plasma diluted to 80% with isotonic sodium phosphate buffer (pH 7.4) as previously described (Di Stefano et al. 2006). The prodrug **1** showed good stability toward gastrointestinal hydrolysis, being the half time for chemical hydrolysis in SGF of ca. 20 h and twice as much as that in isotonic sodium phosphate buffer, thus meeting the requirements for gastrointestinal absorption after oral administration (Table 2). On the contrary, in rat and human

Table 1 Physicochemical properties of prodrug **1**

Solubility[a] (mg/mL)				Lipophilicity[a]		
Water	Buffer solutions			log P_{app}	clog *P*	log k_0
	pH 1.3	pH 5.0	pH 7.4			
6.69 (±0.21)	5.38 (±0.27)	5.00 (±0.22)	4.54 (±0.16)	−0.25 (±0.02)	−0.90 (±0.88)	1.21 (±0.04)

[a] Values are means of three experiments, standard deviation is given in parentheses

Table 2 Rate constants for the chemical and enzymatic hydrolysis of prodrug **1** at 37°C

	Chemical hydrolysis[a]		Enzymatic hydrolysis[a]	
	pH 1.3	pH 7.4	Rat plasma	Human plasma
$t½$ (min)	1240 (±50)	648 (±24)	4.74 (±0.12)	7.32 (±0.30)
k_{obs} (min^{-1})	5.67 (±0.17) 10^{-4}	1.07 (±0.05) 10^{-3}	0.15 (±0.01)	0.10 (±0.01)

[a] Values are means of three experiments, standard deviation is given in parentheses

plasma, liquid chromatography/mass spectrometry (LC/MS) measurements on extracts, performed by using the previously proposed LC/MS/MS method and electrospray ionization (ESI) mode for MS experiments (Sozio et al. 2010), pointed out a rapid bioconversion of prodrug **1** into LD.

Pharmacological studies were performed to test the ability of prodrug **1** to increase basal levels of striatal DA and LD and therefore affecting brain neurochemistry associated with dopaminergic activity. Figures 2 and 3 show plasma concentrations–time profiles of LD and DA striatal levels, respectively, in rats following oral administration by intragastric tube of prodrug **1,** LD itself or tripeptide **5**. With regard to basal levels of DA (1.5 ng/mg tissue), prodrug **1** elicited a sustained release compared to that induced by LD ($P < 0.05$). The effect reached its maximum 4 h after administration, and maintained higher levels of striatal DA, compared to those induced by LD, from the third to the twelfth hour following the treatment. The values of LD plasma concentration 4 h post dose were ca. 110 μg/ml for prodrug **1** and 75 μg/ml for LD, with rapid decrease of plasma levels 2 h after administration in the latter case. The prolonged plasma LD levels induced by prodrug **1** with respect to equimolar doses of LD could be beneficial in the treatment of motor fluctuation in advanced state of the disease because they prevent the well known wearing-off phenomenon (or end-of-dose deterioration) directly related to LD plasma level fluctuation after long-term treatment of PD with chronic LD therapy (Koller et al. 1999). This feature of prodrug **1** administration is even more intriguing if one considers the relatively short half-life of prodrug **1** in plasma (from 4 to 5 min in rat plasma and from 7 to 8 min in human plasma, see Table 2). Furthermore, the plasma concentration profiles of Figs. 2 and 3 are consistent with a slow release of LD in the brain afterwards **1** administration and may represent a desirable therapeutic feature in order to reduce the frequency of drug administration.

In the present study, we assessed also the antioxidant efficacy of prodrug **1** and tripeptide **5** by using two different assays, the DPPH–HPLC and the DMSO competition methods. This activity could be efficaciously exploited in therapy as potential neuroprotective agent. The 1,1-diphenyl-2-picrylhydrazyl (DPPH) has a characteristic

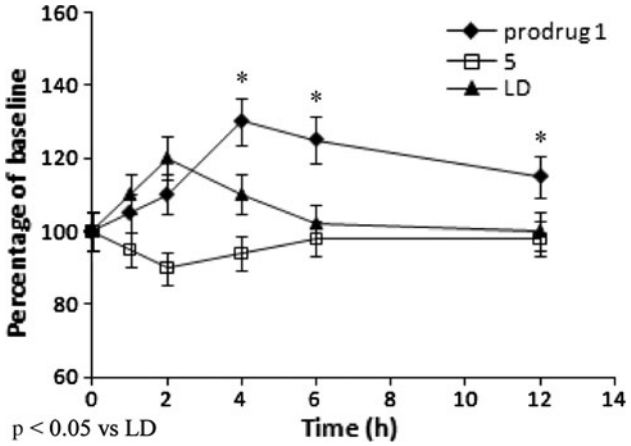


Fig. 2 Effect of oral administration of compound **5**, LD or prodrug **1** (0.332 mmol/kg) on DA striatal levels

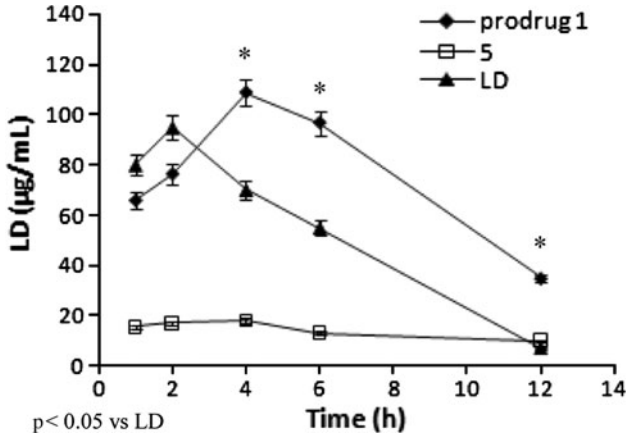


Fig. 3 Effect of oral administration of compound **5**, LD or prodrug **1** (0.332 mmol/kg) on LD plasma levels

absorption at 517 nm whose peak area decreases if the tested compound is a free radical scavenger and, by reacting with DPPH, converts it to the stable diamagnetic molecule 1,1-diphenyl-picrylhydrazine (Panteleon et al. 2008). Since the decrease in the peak depends on the concentration of the tested compound, four different concentrations were investigated (50, 100, 300 and 600 μM). Our results pointed out that the radical scavenging activity of the investigated compounds increased with concentration (Fig. 4) and with incubation time (data not shown). Moreover, tripeptide **5** showed a stronger activity than

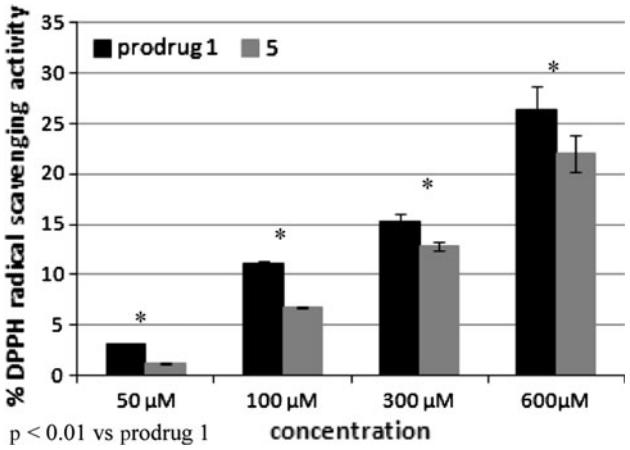


Fig. 4 % DPPH radical scavenging activity after 20 min incubation

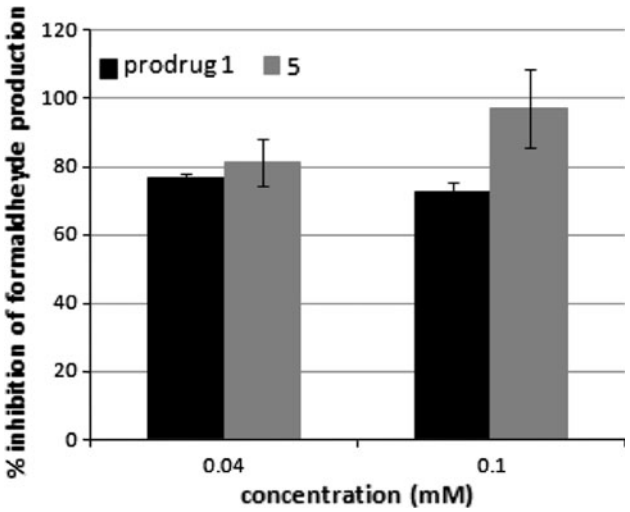


Fig. 5 Percentage of prodrug **1** or compound **5** competition with DMSO for hydroxyl radical

prodrug **1** ($P < 0.01$), thus indicating that the LD moiety in **1** can show its pro-oxidant activity and may thus affect the prevailing antioxidant activity of the tripeptide **5** (Smith et al. 1994).

The competition of our prodrug **1** or tripeptide **5** with DMSO for·the hydroxyl radical generated by the Fe^{3+}/ascorbic acid system (Melagraki et al. 2009; Burini and Coli 2004), expressed as percent of inhibition of formaldehyde production, was used as well for the evaluation of their hydroxyl radical scavenging activity (see Fig. 5). Both the prodrug **1** and the tripeptide **5** compete with DMSO for the hydroxyl radical and reduce about 80% production of formaldehyde at the concentrations of 0.04 and 0.1 mM. This evidence showed that the modification of the GSH moiety, obtained by replacing the cysteine GSH portion with methionine, is responsible for the hydroxyl radical scavenging activity.

In conclusion, we have synthesized a new peptidomimetic prodrug constituted by a modified GSH, as the carrier towards CNS, and LD methylester, as the active antiparkinson agent. This compound demonstrated to cross unaltered the acidic environment of the stomach, to be stable enough to be absorbed from the intestine, to have radical scavenging activity and to release LD in human plasma after enzymatic hydrolysis. Compared to other drugs intended for PD therapy, prodrug **1** appears to be particularly promising because it showed to induce sustained delivery of DA in rat striatal tissue with respect to equimolar administration of LD itself. Taken together, these results are of significance for prospective therapeutic application of prodrug **1** in pathological events associated with free radical damage and decreasing DA concentration in the brain.

Acknowledgments Financial support from Ministero dell' Istruzione, dell'Università e della Ricerca (MIUR) is gratefully acknowledged.

Conflict of interest The authors declare that they have no conflict of interest.

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