Bioorganic & Medicinal Chemistry 18 (2010) 1834–1843

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Design, synthesis, and preliminary pharmacological evaluation of new imidazolinones as L-DOPA prodrugs

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ARTICLE INFO

Article history:
Received 2 October 2009
Revised 13 October 2009
Accepted 19 January 2010
Available online 25 January 2010

Keywords:
L-DOPA prodrug
Parkinson's disease
Dopamine release
DPPH

ABSTRACT

L-DOPA, the immediate biological precursor of dopamine, is still considered the drug of choice in the treatment of Parkinson's disease. However, therapy with L-DOPA is associated with a number of acute problems. With the aim to increase the bioavailability after oral administration, we designed a multi-protected L-DOPA prodrugs able to release the drug by both spontaneous chemical or enzyme catalyzed hydrolysis. The new compounds have been synthesized and preliminarily evaluated for their water solubility, log *P*, chemical stability, and enzymatic stability. The results indicate that the incorporation of the amino acidic moiety of L-DOPA into an imidazoline-4-one ring provides prodrugs sufficiently stable to potentially cross unchanged the acidic environment of the stomach, and to be absorbed from the intestine. They also might be able to release L-DOPA in human plasma after enzymatic hydrolysis. The ability of prodrugs **6a–b** to increase basal levels of striatal DA, and influence brain neurochemistry associated with dopaminergic activity following oral administration, as well as the radical-scavenging activity against DPPH for compounds **6a–b** and **15a** are also reported.

1. Introduction

The aging of the population in industrialized countries suggests that neurodegenerative diseases such as Parkinson's, Alzheimer's, and Huntington's diseases, will be epidemical in the next decades.

Parkinson's disease (PD) is a neurodegenerative disorder associated primarily with loss of dopamine (DA) neurons in the nigrostriatal system.[1] Current therapy for PD is essentially symptomatic, and L-DOPA (**1a**), the immediate biological precursor of DA, is still considered the drug of choice in the treatment of PD. Substitution therapy with L-DOPA is, however, associated with a number of acute problems. The drug undergoes extensive decarboxylation to DA by amino acid decarboxylase (AADC) in the gastrointestinal tract before entering the systemic circulation and is converted by catechol-*O*-methyltransferase (COMT) into the inactive metabolite 3-*O*-methyldopa before crossing the blood–brain barrier.

The peripheral conversion of L-DOPA to DA is responsible for the typical gastrointestinal (nausea, emesis) and cardiovascular (arrhythmia, hypotension) side effects. To minimize the conversion to DA outside the central nervous system (CNS), L-DOPA is usually administrated in combination with peripheral inhibitors of AADC (carbidopa and benserazide). In spite of that, other central nervous side effects such as dyskinesia, on–off phenomenon and end-of-dose deterioration still remain. These effects might be reduced by attenuating peaks and rapid fluctuations of L-DOPA plasma levels.[2,3]

The drug's physicochemical properties such as low water and lipid solubility, and the high susceptibility to chemical and enzymatic degradation are the main factors responsible for the poor bioavailability and the wide range of inter- and intra-patient variations of plasma levels. Moreover it should be taken into account that only 1% of the daily orally administered dose of L-DOPA is able to reach the CNS. The bioavailability, after oral administration, is only 33%, being limited by metabolic instability, low water solubility (1.8 mg/mL), and low solubility in lipids (log $P = -2.38$). In order to improve the bioavailability the prodrug approach appeared to be promising. Several L-DOPA prodrugs have been prepared over the past decades in an effort to overcome these problems.[4]

An ideal L-DOPA prodrug should be soluble in water and in lipids, completely absorbed by the gastrointestinal tract without any chemical degradation or metabolism, and thus deliver intact L-DOPA in the blood stream at a reproducible therapeutic level.

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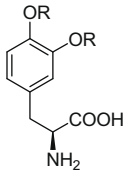
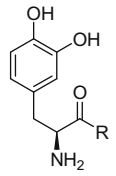
doi:10.1016/j.bmc.2010.01.041

Since the first example of an L-DOPA prodrug designed by Bodor and colleagues, several compounds have been developed including phenol esters (**1b–c**) and carboxylic esters (**2a–b**), amides and peptides (**3a–b, 4a–c**). Most of these compounds show higher bioavailability than L-DOPA and higher L-DOPA/DA ratio in plasma. In particular, dipeptides **4a–c** are reported as L-DOPA prodrugs showing higher bioavailability and lower toxicity than L-DOPA.[5]



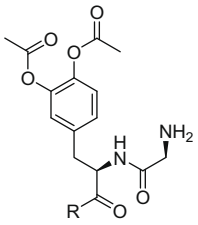
1

a R=H
b R=COMe
c R=COCMe$_3$



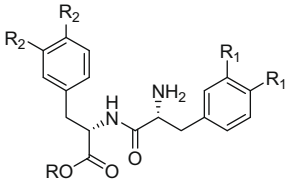
2

a R=OMe
b R=OCH$_2$Ph



3

a R=OH
b R=OMe

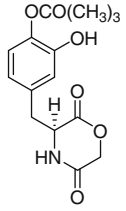


4

a R=H; R$_1$=R$_2$=OCOMe
b R=Me; R$_1$=R$_2$=OCOMe
c R=CH$_2$Ph; R$_1$=R$_2$=OCOMe

It has been demonstrated that enteric adsorption of amino acids combined in dipeptides is faster than the adsorption of single amino acids administered as their mixture due to the presence of dipeptide- and tripeptide-specific carrier systems. It has been proposed that such carrier systems might ameliorate the enteric adsorption of drugs through their dipeptide derivatives.[6]

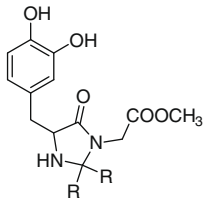
Multi-protected L-DOPA prodrugs have shown better results considering L-DOPA delivery in the blood. In a previous work, we designed and synthesized the L-3-(3-hydroxy-4-pivaloyloxybenzyl)-2,5-diketomorpholine **5** as L-DOPA prodrug.[7]



5

In order to obtain a lipophilic prodrug with enhanced absorption and to protect the L-DOPA amino acid moiety toward decarboxylation by AADC, the carboxylic and amino groups of compound **5** were masked in a 2,5-diketomorpholine skeleton as amide and ester groups, respectively. Moreover, the catechol was protected by the pivaloyl group considering that L-3-(3-hydroxy-4-pivaloyloxyphenyl)alanine produces a sustained L-DOPA plasma level and large L-DOPA bioavailability after oral dosing in rats and dogs.[8] The prodrug **5** met the requirements for gastrointestinal absorption, showed good stability toward gastrointestinal hydrolysis, and released L-DOPA in human plasma after enzymatic hydrolysis. Thus the 2,5-diketomorpholine ring seems to be useful in the design of L-DOPA prodrugs.

Following these studies, a new multi-protected L-DOPA prodrug (**6a–b**) was considered in which the amino acidic functional group is included in an imidazolinone ring. A similar strategy was previously reported for the protection of enkephalins from aminopeptidase degradation.[9] It was demonstrated that such modification is able to protect the enkephalins N-terminal amino acid residue from plasmatic and mucosal enzymatic degradation.[10] Moreover, the imidazoline-4-one is promptly reverted to the original peptide at the physiological pH and temperature conditions with a rate of hydrolysis strongly dependent upon the steric hindrance of the 2-position substituents. In addition, imidazoline-4-one derivatives showed higher lipophilicity than the original peptide, and lipophilicity was modulated by changing the nature of the 2-position substituents. These enkephalin prodrugs showed increased penetrability in a blood–brain barrier model.[11]



6

a R=Me
b R=Et

Based on the increased enzymatic stability and improved blood–brain barrier penetration induced by the imidazoline-4-one ring we designed **6** as potential L-DOPA prodrugs. Herein, in this report, we describe the details of the synthesis and preliminary pharmacological investigations of these compounds.

2. Chemistry

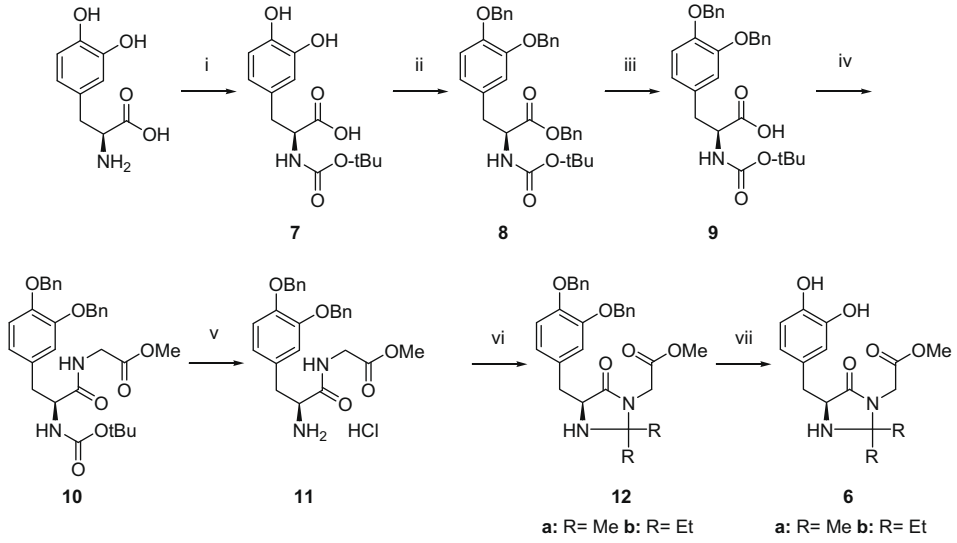
In order to prepare imidazoline-4-one prodrugs, L-DOPA was conjugated with glycine to obtain a dipeptide suitable for its transformation to the 2,2,-imidazoline-4-one derivative. The target molecules **6ab** were obtained following the synthetic pathway reported in Scheme 1.

Thus, the L-DOPA amino group was reacted with *tert*-butyl dicarbonate to give **7**.[7] By treatment of **7** with benzyl bromide under basic condition, **8**[7] was obtained and its basic hydrolysis gave acid **9**.[7] By reaction of **9** with glycine methyl ester hydrochloride, **10** was obtained. The BOC protecting group was removed under acidic conditions and the obtained **11** treated with the proper ketone to give **12** that promptly afforded the target compounds **6a–b** after catalytic hydrogenation.

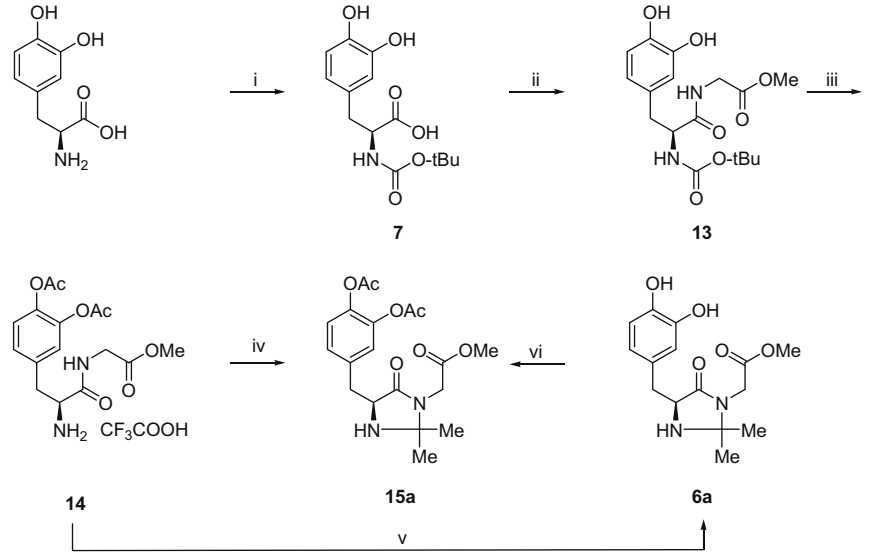
O-Acyl derivatives of phenolic and catecholic drugs represent a well known prodrug approach.[12] O-Acylation may be able to increase lipophilicity as well as to improve stability against COMT metabolism of catecholic drugs. Thus, in order to evaluate the effect of acetylation on prodrug **6a**, compound **15a** was synthesized following the pathway reported in Scheme 2. Such a pathway may also represent a shorter alternative for the synthesis of compound **6a**.

Briefly, by reaction of **7** with glycine methyl ester hydrochloride, compound **13** was obtained. It was reacted with acetyl chloride in TFA to give the diacetyl derivative **14**. Treating **14** with the proper ketone under basic conditions afforded the *S* enantiomer **15a**. Control of basicity seems to be crucial in the latter step. In fact when the pH value was higher than 8, compound **6a** was directly obtained.

The asymmetric carbon of the molecules has never been involved in the reaction mechanisms so its stereochemistry should not be affected by the performed reactions. However, to confirm



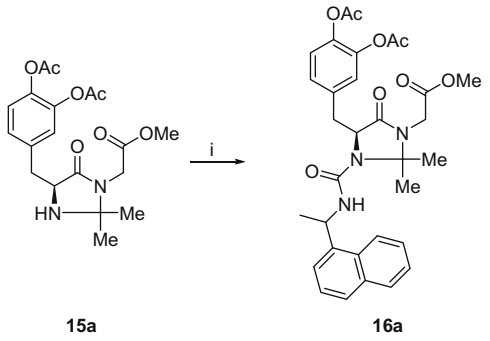
Scheme 1. Reagents: (i) $(BOC)_2O$; (ii) BnBr, K_2CO_3, dioxane–H_2O; (iii) 1 N NaOH; (iv) $H_2NCH_2COOMe.HCl$, EDCI, HOBT, TEA, DCM; (v) MeOH, HCl; (vi) $R_2C{=}O$, MeOH, NaOH; (vii) H_2, 10% Pd/C, EtOH.



Scheme 2. Reagents and conditions: (i) $(BOC)_2O$; (ii) $H_2NCH_2COOMe{\cdot}HCl$, EDCI, HOBT, TEA, DCM; (iii) AcCl, TFA; (iv) Me_2CO or Et_2CO, MeOH, NaOH, pH 8; (v) NaOH, MeOH, pH >8; (vi) AcCl, TFA.

such a statement we also prepared the racemate [i.e., (±)**15**] starting from DL-DOPA and then, treating both **15a** and its racemate (±)**15** with (*R*)-(−)-1-(1-naphtyl)-ethyl isocyanate to give the derivatives **16a** (Scheme 3) and the corresponding racemate, respectively. As expected, comparing the HPLC chromatograms of **16a** and its racemate, no racemization was observed after the described reactions.

The same HPLC profile was displayed for **16a** synthesized from **15a** which was obtained treating the catechol **6a** with acetyl chloride in TFA, prepared following the synthetic pathway reported in Scheme 1. Such a result confirmed, once again, that the stereochemistry of the asymmetric carbon was retained.



Scheme 3. Reagents: (i) (*R*)-(−)-1-(1-naphtyl)-ethyl isocyanate, $CHCl_3$.

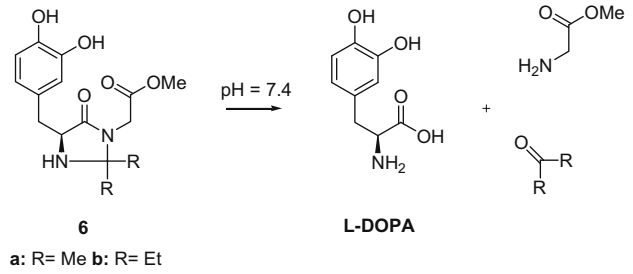
3. Results and discussion

The physicochemical properties, water solubility and octanol/water partition coefficients, were determined for compounds **6a–b** and **15a** (see Table 1). In fact, the apparent partition coefficient (log *P*) may be used to predict the distribution of a drug in a biological system and may be correlated to other factors such as adsorption, distribution and CNS penetration.[13]

Table 1
Measured water solubility and log *P* values

Compound	Water solubility[a] (mg/mL)	Log *P*[a]
6a	3.99 (±0.46)	10.1×10^{-2} ($\pm 0.3 \times 10^{-2}$)
6b	0.26 (±0.04)	94×10^{-2} ($\pm 4 \times 10^{-2}$)
15°	26.04 (±0.76)	4.2×10^{-2} ($\pm 0.1 \times 10^{-2}$)
L-DOPA	3.60	−2.38

[a] The reported values are the means of three experiments. The SEM are reported in parenthesis.



Scheme 4.

The results indicate that both **6a** and **6b**, as well as compound **15a**, are more lipophilic than L-DOPA. Compound **6a** displays water solubility comparable to L-DOPA whereas **6b** is one order of magnitude less water soluble. In such a test, **15a** shows the highest water solubility compared to **6a**, **6b**, and L-DOPA. However, assuming a passive transport through a biological membrane, it is reported that for good intestinal absorption after oral administration a log $P \geqslant 1.35$ is required. More lipophilic compounds (log $P \geqslant 2$) are instead required for optimum CNS penetration.[13] Although, comparing the log *P* values of prodrugs **6a–b** and **15a** with those of L-DOPA, an improvement has been achieved, it does not suggest good absorption after oral administration, and suggests further structural modification in order to increase their lipophilicity.

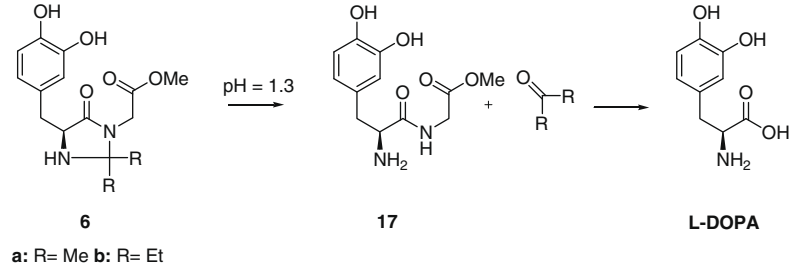
The chemical stability of prodrugs **6a–b** and **15a** was assayed in vitro at 37 °C in aqueous buffer solutions of pH 1.3 (nonenzymatic simulated gastric fluid) and pH 7.4. The products of the reaction were characterized.[14] When the hydrolysis was performed in 0.02 M phosphate buffer (pH 7.4), prodrugs **6a–b** released directly L-DOPA (Scheme 4), whereas reaction proceeds through intermediate **17** when a hydrochloric acid buffer of pH 1.3 was used (Scheme 5). Concerning the chemical stability of **15a**, in both buffer solutions the prodrug was hydrolyzed to compound **6a**.

The kinetics of chemical hydrolysis was determined (Fig. 1). The results indicate that **6a** is the most stable tested compound at pH 1.3 in the series. Compounds **6a–b** show comparable stability at pH 7.4 (Table 2). However, it was observed that the catechol acetylation of **6a** increases the stability at such a pH value. Thus, the high half time values indicate a considerable chemical stability of the new prodrugs at both the pH conditions examined.

The stability of compounds **6a–b** and **15a** in aqueous buffer solution of pH 1.3 implies that they might pass unchanged through the stomach after oral administration.

The enzymatic stability of the new prodrugs was also studied at 37 °C in 80% rat and in 80% human plasma (Table 3). The first-order kinetic plots for hydrolysis of the new prodrugs is reported in Figure 2.[14]

Compound **6a**, undergoes faster hydrolysis than **6b** in rat plasma, but not in human plasma, where comparable hydrolysis rates were observed. It can be noticed that the prodrug **15a** is cleaved to L-DOPA with the fastest rate of hydrolysis in the series, being hydrolyzed more quickly in rat plasma than in human plasma. However, the formation of L-DOPA, unexpectedly, does not pass through the formation of the prodrug **6a**.



Scheme 5.

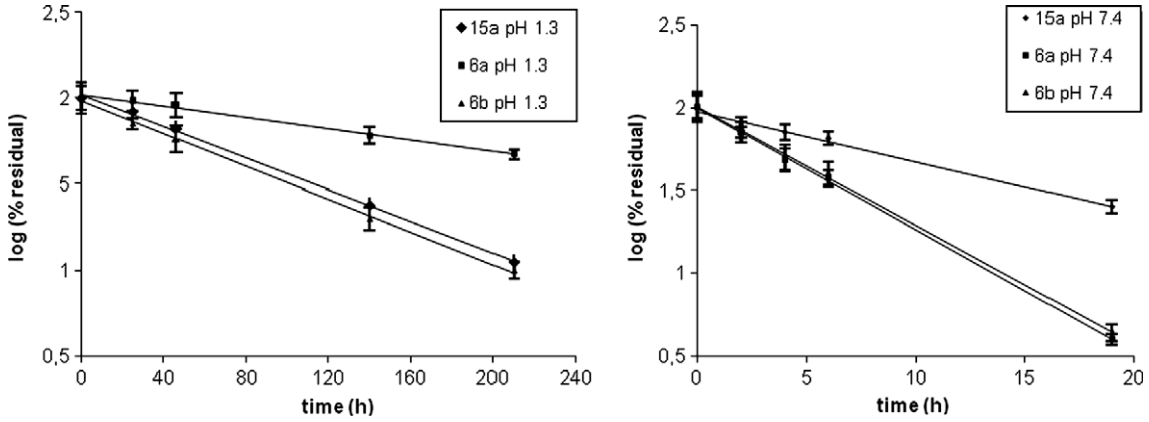


Figure 1. First-order kinetic plots for hydrolysis of prodrugs **6a–b** and **15a** in hydrochloric acid buffer of pH 1.3 and in phosphate buffer of pH 7.4 at 37 °C.

Table 2
Kinetic data for chemical hydrolysis of prodrugs **6a–b** and **15a** at 37 °C

Compound	pH 1.3[a]		pH 7.4[a]	
	$t_{1/2}$ (h)	K_{obs} (h^{-1})	$t_{1/2}$ (h)	K_{obs} (h^{-1})
6	189.33 (±0.95)	0.0037 ($\pm 1.9 \times 10^{-4}$)	4.20 (±0.38)	0.17 ($\pm 1.5 \times 10^{-2}$)
6b	64.32 (±1.29)	0.011 ($\pm 2.2 \times 10^{-4}$)	4.75 (±0.24)	0.14 ($\pm 7.1 \times 10^{-3}$)
15a	71.24 (±0.85)	0.01 ($\pm 0.39 \times 10^{-3}$)	11.55 (±0.29)	0.06 ($\pm 1.5 \times 10^{-3}$)

[a] The reported values represent the mean of three experiments. The SEM are reported in parenthesis.

Table 3
Rate constants for the hydrolysis of prodrugs **6a–b** and **15a** in 80% rat plasma and in 80% human plasma at 37 °C

Compound	Rat plasma[a]		Human plasma[a]	
	$t_{1/2}$ (min)	K_{obs} (min^{-1})	$t_{1/2}$ (min)	K_{obs} (min^{-1})
6a	70.2 (±1.4)	0.009 ($\pm 0.2 \times 10^{-3}$)	42.82 (±2.14)	0.017 ($\pm 0.8 \times 10^{-3}$)
6b	113.2 (±5.7)	0.006 ($\pm 0.3 \times 10^{-3}$)	47.56 (±2.85)	0.015 ($\pm 0.9 \times 10^{-3}$)
15a	3.9 (±0.3)	0.175 ($\pm 1.4 \times 10^{-2}$)	23.50 (±1.20)	0.029 ($\pm 0.9 \times 10^{-3}$)

[a] The reported values represent the mean of three experiments. The SEM are reported in parenthesis.

Taken together, these results indicate that the incorporation of the amino acidic moiety of L-DOPA into an imidazoline-4-one ring provides prodrugs sufficiently stable to potentially cross unchanged the acidic environment of the stomach, and to be absorbed from the intestine. They also might be able to release L-DOPA in human plasma after enzymatic hydrolysis. However, further modifications of the prodrug molecules designed to enhance their lipophilicity seems to be required in order to increase the possibility of good intestinal adsorption.

Further biochemical and pharmacological studies were conducted to test the ability of prodrugs **6a–b** to increase basal levels of striatal DA, and influence brain neurochemistry associated with dopaminergic activity, following oral administration (Fig. 3). Prodrug **15a** was not examined due to its extremely short half life in rat plasma.

With regard to basal levels of striatal DA, compound **6a** elicited a long-lasting effect compared to that induced by L-DOPA, even if it was lower after the first hour. This effect reached its maximum 4 h post-administration, and maintained higher levels of striatal DA, compared to L-DOPA, from the second to the fifth hour following administration. Such a profile is consistent with a slow release of L-DOPA to the brain; this might represent a desirable therapeutic feature for reducing the frequency of drug administration. The measurement of basal levels of the metabolite 3,4-dihydroxyphenyl acetic acid (DOPAC) confirms the previous statement.

L-DOPA usually undergoes decarboxylative and oxidative metabolism prior to 3-O-methylation. However, with prodrugs **6a–b**, protection of the amino acidic moiety of the molecule seems to invert the order of the metabolic reactions and 3-O-methylation might occur first. Such an hypothesis may explain the unexpected profile (Fig. 3) shown by the curve of increment of the basal levels of the metabolite homovanillic acid (HVA). 3-*O*-Methyl derivatives are more lipophilic than their parent catechol prodrugs thus they can more easily reach the brain where they are expected to undergo hydrolysis, decarboxylation, and oxidation to the final HVA.[15] As a consequence, compared to L-DOPA, higher levels of HVA after only one hour post-administration of the prodrugs **6a–b** were detected. Finally, due to the long-lasting effect of prodrugs **6a–b**, HVA levels are maintained higher for a longer time than after the administration of L-DOPA.

It has been reported that chronic oxidative stress induces progressive, irreversible damage affecting the normal physiological functions of the CNS, and seems to play a crucial role in several CNS diseases, including PD.[16]

Many phenolic compounds have been reported as antioxidants with their antioxidant activity related to their structure.[17] Moreover, it has been reported that L-DOPA oxidation products prevented H_2O_2-induced oxidative damage to cellular DNA in cultured tissue cells.[18] At the same time, catecholamine metabolism by monoamine oxidase (MAO) leads to the formation of H_2O_2, which can be converted into the more reactive hydroxyl radicals through interaction with ferrous iron.[19] The reaction involving H_2O_2, iron, and DA may be a source of a dopaminergic neurotoxin such as 6-hydroxydopamine which seems to represent a contributory factor in the etiopathogenesis of PD.[20] Thus, free radical-scavenging compounds could work as adjuvant tools for the treatment of such a neurodegenerative disease.

The DPPH (diphenyl-1-picrylhydrazyl) radical assay is one of the widely used detection procedures, which facilitates analysis of various natural antioxidants, especially phenols.[21] Thus the radical-scavenging activity against DPPH was evaluated for compounds **6a–b** and **15a** and for vitamin E, caffeic acid and L-DOPA as reference compounds (Fig. 4). From the results it can be observed that compound **6b** displays high antioxidant activity, comparable to that of the reference compounds, even at low concentration. The diacetyl

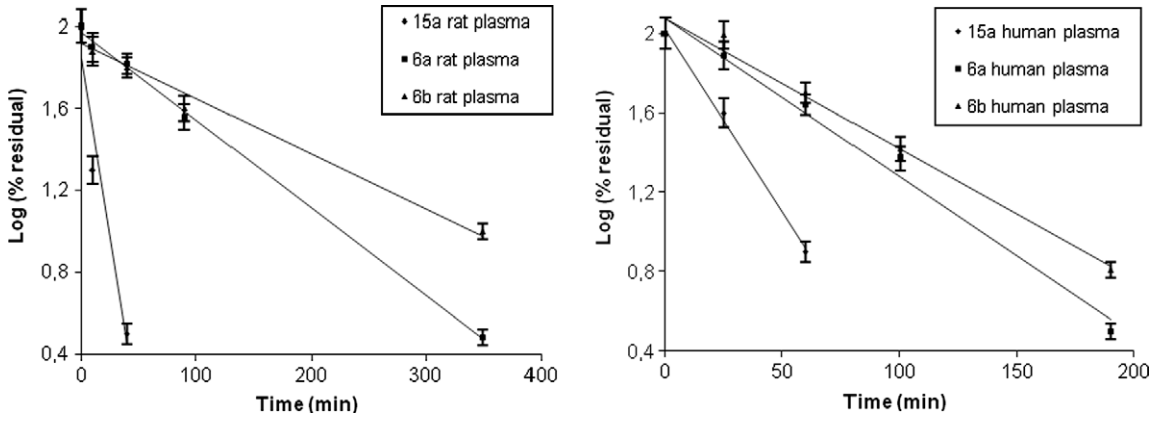


Figure 2. First-order kinetic plots for hydrolysis of prodrugs **6a–b** and **15a** in 80% rat and human plasma at 37 °C.

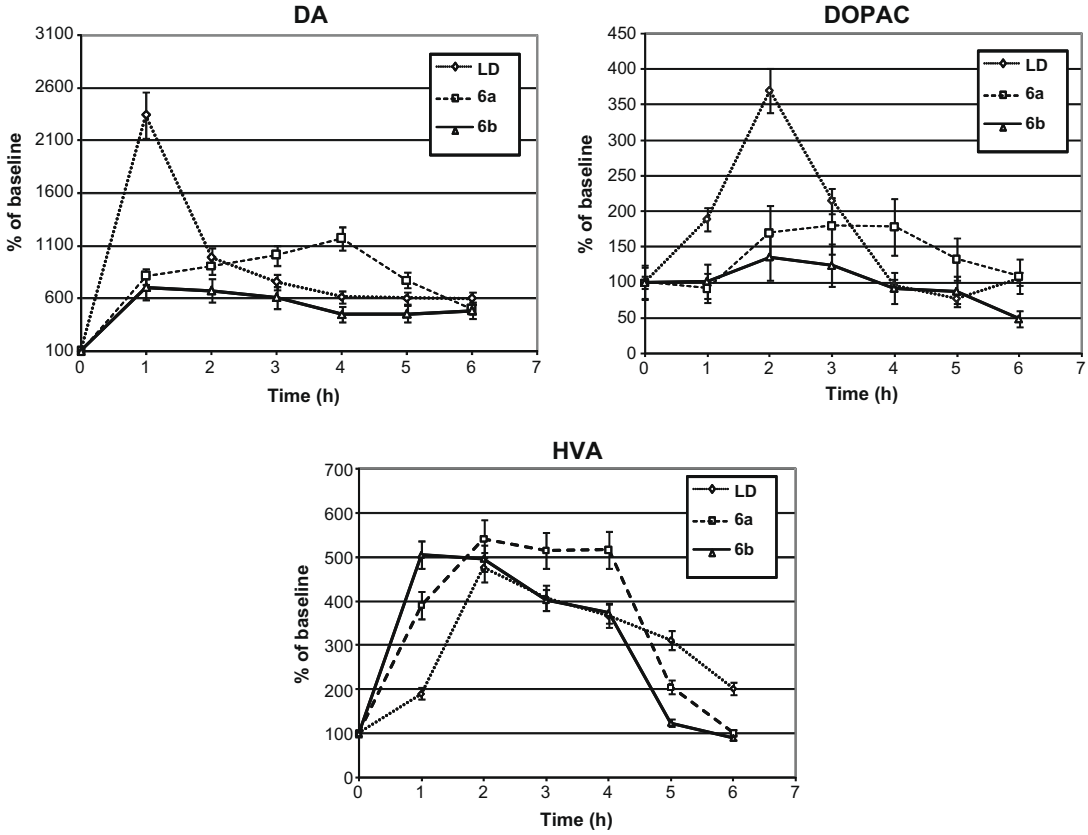


Figure 3. Effect of oral administration of L-DOPA and prodrugs **6a–b** (0.33 mmol/kg) on striatal baseline dialysate of DA, DOPAC, and HVA.

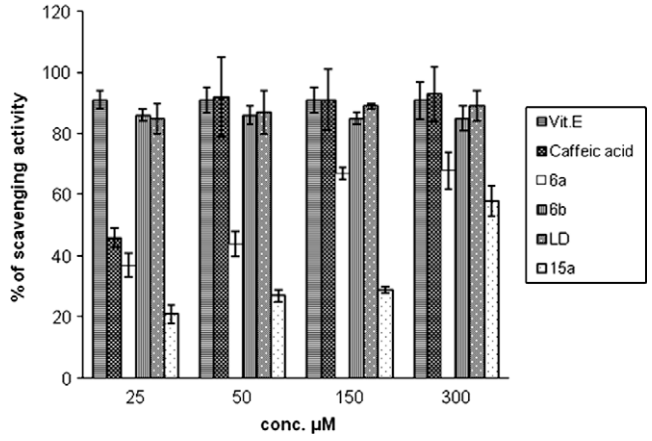


Figure 4. Scavenging activity of prodrugs **6a–b** and **15a** with vitamin E (Vit. E), caffeic acid and L-DOPA (LD) as controls.

derivative **15a** proved to be the least active member of the series, probably because it lacks a catecholic or phenolic function. Watanabe et al.,[22] studying the antioxidant activity of a series of arylimidazolinones, concluded that the activity of their compounds was greatly affected by their lipophilicity. Such an observation might explain why compound **6a**, although structurally similar **6b**, shows much lower antioxidant activity.

4. Conclusion

In summary, we have synthesized a new L-DOPA prodrug scaffold based on an imidazolinone nucleus. These catecholic compounds might be able to cross the acidic environment of the stomach unchanged, are stable enough to be absorbed from the intestine, and release L-DOPA in human plasma after enzymatic hydrolysis. However, work is currently in progress to determine whether an increase in lipophilicity might be useful to ameliorate the possibility of their absorption after oral administration and to evaluate their distribution into the CNS.

5. Experimental

5.1. Chemistry

5.1.1. General procedures

Melting points were measured on a Büchi 510 apparatus and are uncorrected. Microanalyses were performed on a 1106 Carlo Erba CHN Analyzer. Analyses were within ±0.4% of the calculated values. HPLC analysis and mass spectra were recorded on a HPLC-DAD-RID-MS (ion trap) 1100—LC MSD trap sl Agilent Technologies. HPLC analysis for compound **16** was performed on HP Series II 1090 equipped with diode array detector set at 254 nm. Column: Phenomenex Gemini-NX 5μ C_{18} 110 Å 250 × 4.6 mm. Eluant: 40:60 water/methanol. Flow 1 mL/min.

^{1}H NMR spectra were recorded on a Varian VXR 200-MHz spectrometer. Chemical shift values are reported in parts per million (δ) downfield from the internal standard tetramethylsilane (Me_4Si). The identity of all new compounds was confirmed both by elemental analyses and NMR data. MS analysis was performed only to confirm the identity of the final products. TLC was performed with Merck 60 F_{254} precoated silica on glass. Solutions were routinely dried over anhydrous sodium sulfate prior to evaporation. Chromatographic purification was performed by Merck 60 70–230 mesh ASTM silica gel columns with the reported solvent.

5.1.2. (*S*)-2-(*tert*-Butoxycarbonylamino)-3-(3,4-dihydroxy phenyl)-propionic acid (7)

To an ice cooled solution of L-DOPA (1 g, 5 mmol) and NEt_3 (1.08 mL, 7.5 mmol) in dioxane/water (50%, 20 mL), di-*tert*-butyldicarbonate (1.2 g, 5.5 mmol) was added portionwise. The resulting mixture was stirred for 30 min at 0 °C, then at rt for 18 h. The solution was concentrated under reduced pressure, then water (10 mL) and ethyl acetate (10 mL) were added. The aqueous layer was washed with ethyl acetate, acidified to pH 1 with 1 N HCl, and back-extracted with ethyl acetate. The organic extracts were washed with brine, dried over Na_2SO_4, and evaporated to give the protected amino acid, *N*-Boc-L-DOPA, as a brown oil which was used in the next step without further purification.

5.1.2.1. Data for (*S*) enantiomer. Yield 95%. $[\alpha]_D^{20} = +12.75$ (*c* 1, MeOH). ^{1}H NMR ($CDCl_3$): δ 7.80 (br s, 1H, COOH); 6.90–6.60 (m, 2H, ArH); 6.50 (d, 1H, ArH); 5.20 (d, 1H, NH); 4.60–4.25 (m, 1H, CH); 2.90 (s, 2H, CH_2); 1.35 (s, 9H. $3CH_3$) Anal. ($C_{14}H_{19}NO_6$): C, H, N.

5.1.2.2. Data for racemate. Yield 88.5%. ^{1}H NMR (DMSO-d_6): δ 12.50 (br s, 1H, COOH); 8.70 (br s, 2H, 2OH); 7.00 (d, 1H, ArH); 6.60–6.35 (m, 3H, 2ArH and NH); 4.00–4.85 (m, 1H, CH-N); 2.80–2.40 (m, 2H, CH_2); 1.35 (s, 9H. CH_3).

5.1.3. (*S*)-Benzy-3-(3,4-bis-benzyloxyphenyl)-2-(*tert*-butoxy carbonylamino) propanoate (8)

A mixture of **S**-(**7**) (2.25 g, 7.6 mmol), K_2CO_3 (1.66 g, 12 mmol), and benzyl chloride (4.06 g, 23.7 mmol) in acetone (30 mL) was refluxed for 5 h. The solvent was evaporated under reduced pressure and the residue was diluted with water and then extracted with ethyl acetate. The organic extracts were washed with brine, dried over Na_2SO_4 and evaporated. The residue was recrystallized from ethyl acetate/hexane to give the product. Yield 97%. Mp 114–116 °C. $[\alpha]_D^{20} = +3.2$ (*c* 1, $CHCl_3$). ^{1}H NMR ($CDCl_3$): δ 7.45–7.22 (m, 10H, ArH), 6.78 (d, 1H, ArH), 6.70 (d, 1H, ArH), 6.55 (d, 1H, ArH), 5.05 (s, 2H, CH_2), 5.02 (s, 2H, CH_2), 5.00 (s, 2H, CH_2), 4.95 (d, 1H, NH), 4.58 (m, 1H, CH), 2.98 (d, 2H, CH_2), 1.40 (s, 9H, $3CH_3$). Anal. ($C_{35}H_{37}NO_6$,) C, H, N.

5.1.4. (*S*)-3-3,4-Bis(benzyloxy)phenyl-2-(*tert*-butoxy carbon ylamino)propanoic acid (9)

To a suspension of **8** in water/dioxane (8.25%, 55 mL), 1 N NaOH solution (10 mL) was added dropwise. The mixture was stirred at room temperature for 24 h. The solution was acidified to pH 1 with 2 N HCl then extracted with ethyl acetate. The organic extracts were washed with brine, dried over Na_2SO_4 and evaporated. The residue was recrystallized from CH_2Cl_2/*n*-hexane to give the product. Yield 86%. Mp 125–126 °C. ^{1}H NMR (DMSO-d_6): δ 12.60 (s, 1H, OH), 7.45–7.22 (m, 10H, ArH), 7.10–6.90 (m, 3H, ArH, NH), 6.76 (d, 1H, ArH), 5.05 (s, 4H, $2CH_2$), 4.05 (m, 1H, CH), 2.98–2.65 (m, 2H, $2CH_2$), 1.30 (s, 9H, $3CH_3$). Anal. ($C_{28}H_{31}NO_6$,) C, H, N.

5.1.5. (*S*)-Methyl-2-3-(3,4-bis(benzyloxy)phenyl)-2-(*tert*-butoxy carbonylamino)propanamido acetate (10)

An ice cooled solution of glycine methyl ester hydrochloride (0.27 g, 2.1 mmol) and **9** (1 g, 2.1 mmol) in dichloromethane (20 mL) was treated with NEt_3 (0.9 mL, 6.3 mmol), EDCI (0.48 g, 2.5 mmol) and HOBT (0.34 g, 2.5 mmol). The reaction mixture was stirred at 0 °C for 1 h and then at 25 °C for 17 h. The organic solution was washed with water (1 × 5 mL), 2 N HCl solution (1 × 5 mL), saturated $NaHCO_3$ (1 × 5 mL), and then dried on Na_2SO_4. The solvent was removed under reduced pressure and the residue recrystallized from ethyl acetate to give the product. Yield 75%. Mp 133–135 °C. ^{1}H NMR (DMSO-d_6): δ 8.38 (t, 1H, NH), 7.45–7.25 (m, 10H, ArH), 7.05 (s, 1H, ArH), 6.98–6.75 (m, 3H, ArH, NH), 5.05 (s, 4H, CH_2), 4.08 (m, 1H, CH), 3.95 (m, 2H, CH_2), 3.62 (s, 3H, CH_3), 3.0–2.92 (m, 1H, CH_2), 2.86–2.58 (m, 1H, CH_2), 1.30 (s, 9H, $3CH_3$) Anal. ($C_{31}H_{36}N_2O_7$) C, H, N. ESIMS: *m*/*z* $(M-H)^-$ 547.

5.1.6. (*S*)-Methyl 2-(4-(3,4-bis(benzyloxy)benzyl)-2,2-dimethyl-5-oxoimidazolidin-1-yl)acetate (12a)

A solution of **10** in 10 mL of 3 M hydrogen chloride in methanol (10 mL) was stirred at room temperature for 30 min. The solvent was removed under reduced pressure and the residue (**11**) was dissolved in methanol (10 mL). The pH of the solution was adjusted to 8.2 with 2 N NaOH solution in methanol. Acetone (2 mL) was added, and the mixture stirred at room temperature for 21 h. The solvent was removed under reduced pressure and the residue purified by chromatography on silica gel using ethyl acetate as eluant to afford a colorless oily product. Yield 65%. ^{1}H NMR (DMSO-d_6) δ: 7.52–7.25 (m, 10H, ArH), 7.05 (d, 1H, ArH), 6.95 (d, 1H, ArH), 6.76 (dd, 1H, ArH), 5.10 (s, 4H, $2CH_2$), 3.98 (s, 2H, CH_2), 3.65 (s, 3H, CH_3), 3.60–3.50 (m, 1H, CH), 3.02–2.95 (m, 1H, CH_2), 2.80–2.55 (m, 2H, CH_2, NH), 1.20 (2s, 6H, $3CH_3$). Anal. ($C_{29}H_{32}N_2O_5$) C, H, N. ESIMS: *m*/*z* (MH^+) 489.

5.1.7. (*S*)-Methyl 2-(4-(3,4-dihydroxybenzyl)-2,2-dimethyl-5-oxoimidazolidin-1-yl)acetate (6a)

A mixture of **12a** (1.38 g, 2.8 mmol), and 10% palladium on charcoal (0.140 g), in 25 mL of absolute ethanol (25 mL), under hydrogen atmosphere (20 psi) was stirred at room temperature for 5 h. The mixture was filtered and the solvent was evaporated in vacuo to give a residue purified by column chromatography over silica gel using ethyl acetate as eluant. The residue was recrystallized from ethanol. Yield 75%. Mp 170–172 °C. ^{1}H NMR (DMSO-d_6): δ 8.75–8.65 (2s, 2H, OH), 6.60 (m, 2H, ArH), 6.45 (dd, 1H, ArH), 3.96 (s, 2H, CH_2), 3.72 (s, 3H, CH3), 3.58–3.45 (m, 1H, CH), 2.95–2.86 (m, 1H, CH_2), 2.60–2.42 (m, 1H, CH_2), 120 (2s, 6H, $2CH_3$). Anal. ($C_{15}H_{20}N_2O_5$) C, H, N. ESIMS: *m*/*z* (MH^+) 309.

5.1.8. (*S*)-Methyl-2-(4-(3,4-bis(benzyloxy)benzyl)-2,2-diethyl-5-oxoimidazolidin-1-yl)acetate (12b)

The pH of a solution of **11** hydrochloride (1 g, 2 mmol) in methanol (10 mL) was adjusted to pH 8.2 with 2 N NaOH solution in methanol. 3-Pentanone (1 mL) was added and the mixture was stirred for 24 h at room temperature. The solvent was removed under reduced pressure and the residue was purified by column chromatography on silica gel using a mixture of *n*-hexane/ethyl acetate (80/20, v/v) as eluant. The yellow oil obtained was recrystallized from ethanol. Yield 95%. Mp 170–172 °C. ^{1}H NMR (DMSO-d_6): δ 7.55–7.25 (m, 10H, ArH), 6.98–6.86 (m, 2H, ArH), 6.76 (dd, 1H, ArH), 5.05 (2s, 4H, $2CH_2$), 3.84 (s, 2H, CH_2), 3.76–3.65 (m, 1H, CH), 3.60 (s, 3H, CH_3), 2.96–2.84 (m, 1H, CH_2), 2.72–2.55 (m, 1H, CH_2), 2.20 (br s, 1H, NH), 1.60–1.30 (m, 4H, CH_2), 1.75–1.65 (2t, 6H, $2CH_3$). Anal. ($C_{31}H_{36}N_2O_5$) C, H, N. ESIMS: *m*/*z* (MH^+) 517.

5.1.9. (*S*)-Methyl 2-(4-(3,4-dihydroxybenzyl)-2,2-diethyl-5-oxoimidazolidin-1-yl)acetate (6b)

A mixture of **12b** (0.61 g, 1.2 mmol), 10% palladium on charcoal (0.061 g) in 25 mL of absolute ethanol (11 mL) was stirred under hydrogen atmosphere (25 psi) at room temperature for 24 h. The mixture was filtered and the solvent evaporated under reduced pressure to give a residue that was recrystallized from ethanol. Yield 72%. Mp 153–155 °C.^{1}H NMR (DMSO-d_6): δ 9.88–9.65 (2s, 2H, 2OH), 6.65–6.68 (m, 2H, ArH), 6.45 (dd, 1H, ArH), 4.82 (s, 2H, CH_2), 3.70–3.55 (m, 4H, CH, CH_3), 2.85–2.86 (m, 1H, CH_2), 2.50–2.42 (m, 1H, CH_2), 2.35 (d, 1H, NH), 1.60–1.30 (m, 4H, $2CH_2$), 1.78–1.40 (2t, 6H, $2CH_3$). Anal. ($C_{17}H_{24}N_2O_5$) C, H, N. ESIMS: *m*/*z* (MH^+) 337.

5.1.10. [2-*tert*-Butoxycarbonylamino-3-(3,4-dihydroxyphenyl)-propionylamino]-acetic acid methyl ester (13)

A solution of glycine methyl ester hydrochloride (0.63 g, 5 mmol) and **7** (1.5 g, 5 mmol) in dichloromethane (25 mL) cooled to 0 °C, was treated with DIEA (0.86 mL, 5 mmol); EDCI (0.96 g, 5 mmol) and HOBt (0.67 g, 5 mmol). The reaction mixture was stirred at 0 °C for 1 h and then at 25 °C for 17 h. The organic solution was washed with water (1 × 5 mL); 2 N HCl (1 × 5 mL); saturated $NaHCO_3$ (1 × 5 mL) and then dried over Na_2SO_4. The solvent was removed under reduced pressure and the residue purified by column chromatography using $CHCl_3$/MeOH (95/5 v/v) as eluant to obtain a white glassy solid.

5.1.10.1. Data for (*S*) enantiomer. Yield 62.5%. Mp 125–128 °C (dec.). $[\alpha]_D^{20} = -5.69$ (*c* 1, MeOH). ^{1}H NMR (DMSO-d_6): δ 8.70 (s, 2H, 2OH); 8.38 (t, 1H, NH); 6.85 (d, 1H, ArH); 6.67–6.49 (m, 3H, ArH, NH); 4.08 (m, 1H, CH–N); 3.88 (m, 2H, CH_2); 3.62 (s, 3H, OCH_3); 2.83–2.55 (m, 2H, CH_2); 1.33 (s, 9H, CH_3). Anal. ($C_{17}H_{24}N_2O_7$) C, H, N. ESIMS: *m*/*z* $(M-H)^-$ 367.

5.1.10.2. Data for racemate. Yield 54%. Mp 127–130°C (dec.). ^{1}H NMR (DMSO-d_6): δ 8.70 (s, 2H, OH) 8.38 (t, 1H, NH); 6.90 (d, 1H, ArH); 6.67–6.45 (m, 3H, ArH, NH); 4.08 (m, 1H, CH–N); 3.88 (m, 2H, CH_2); 3.62 (s, 3H, OCH_3); 2.83–2.55 (m, 2H, CH_2); 1.33 (s, 9H, CH_3). Anal. ($C_{17}H_{24}N_2O_7$) C, H, N. ESIMS: *m*/*z* $(M-H)^-$ 367.

5.1.11. 3-(3,4-Diacetoxyphenyl)-1-(2-methoxy-2-oxoethyl amino)-1-oxopropan-2-aminium-2,2,2-trifluoroacetate (14)

To an ice cooled solution of **13** (0.5 g, 1.36 mmol) in trifluoroacetic acid (4 mL), acetyl chloride (0.29 mL, 4.08 mmol) was added dropwise over a period of 5 h, and stirred for 6 h at the same temperature. The reaction mixture was concentrated under reduced pressure (water bath temperature below 15 °C) to give a colorless oil which was triturated in ether, then filtered to give a white foamy solid.

5.1.11.1. Data for (*S*) enantiomer. Yield 54%. Mp 115–117 °C (dec.). $[\alpha]_D^{20} = +13.1$ (*c* 1, MeOH). ^{1}H NMR (DMSO-d_6): δ 9.01 (t, 1H, NH); 8.26 (br s, 3H, NH_3^+); 7.25–7.15 (m, 3H, ArH); 4.16–4.00 (m, 1H, CH–N); 3.96 (d, 2H, CH_2); 3.64 (s, 3H, CH_3); 3.20–2.95 (m, 2H, CH_2); 2.27 (s, 9H, CH_3). Anal. ($C_{18}H_{21}F_3N_2O_9$) C, H, N. ESIMS: *m*/*z* (MH^+) 353.

5.1.11.2. Data for racemate. Yield 56%. Mp 113–116 °C (dec.). ^{1}H NMR (DMSO-d_6): δ 9.07 (t, 1H, NH); 8.26 (br s, 3H, NH_3^+); 7.30–7.21 (m, 3H, ArH); 4.18–4.06 (m, 1H, CH); 3.96 (d, 2H, CH_2); 3.64 (s, 3H, CH_3); 3.20–2.98 (m, 2H, CH_2); 2.27 (s, 9H, CH_3). Anal. ($C_{18}H_{21}F_3N_2O_9$) C, H, N. ESIMS: *m*/*z* (MH^+) 353.

5.1.12. 4-((1-(2-Methoxy-2-oxoethyl)-2,2-dimethyl-5-oxoimid azolidin-4-yl)methyl)-1,2-phenylene diacetate

Method A: **14** (0.5 g, 1 mmol) was dissolved in methanol (10 mL) and the solution was adjusted to pH 8 with a 2 N solution of NaOH in methanol. Acetone (4 mL) was added and the mixture was stirred at rt overnight. The solvent was removed under reduced pressure and the residue was purified by chromatography on silica gel using a mixture of chloroform/methanol (95/5, v/v) as eluant to afford a colorless oil.

Method B: To an ice cooled solution of **6a** (0.02 g, 0.06 mmol) in trifluoroacetic acid (1 mL), acetyl chloride (0.014 mL, 0.19 mmol) was added dropwise over a period of 5 h, and stirred for 6 h at the same temperature. The reaction mixture was concentrated under reduced pressure (water bath temperature below 15 °C) to give a residue which was dissolved in a saturated solution of K_2CO_3 and extracted with ethyl acetate. The organic fraction was dried over sodium sulfate, filtered and evaporated under reduced pressure to give the product.

The composition of the diastereoisomeric mixture (Fig. 2) was determined by HPLC using the column Phenomenex Gemini-NX 5μ C_{18} 110 Å 250 × 4.6 mm with MeOH/H_2O 60:40 as eluant.

5.1.12.1. Data for (*S*) enantiomer (15a). Yield 73% (method A). $[\alpha]_D^{20} = -40.23$ (*c* 0.43, MeOH). ^{1}H NMR (D_2O–DMSO-d_6): δ 7.22–7.08 (m, 3H, ArH); 4.00 (s, 2H, CH_2); 3.70–3.55 (m, 4H, CH and OCH_3); 3.00–2.60 (m, 2H, CH_2); 2.20 (s, 6H, $2CH_3$); 1.25 and 1.20 (2s, 6H, $2CH_3$). Anal. ($C_{19}H_{24}N_2O_7$) C, H, N. ESIMS: *m*/*z* (MH^+) 393.

5.1.12.2. Data for racemate (15). Yield 69% (method A). ^{1}H NMR (D_2O–DMSO-d_6): δ 7.22–7.08 (m, 3H, ArH); 4.00 (s, 2H, CH_2); 3.70–3.55 (m, 4H, CH and OCH_3); 3.00–2.60 (m, 2H, CH_2); 2.20 (s, 6H, $2CH_3$); 1.25 and 1.20 (2s, 6H, $2CH_3$). Anal. ($C_{19}H_{24}N_2O_7$) C, H, N. ESIMS: *m*/*z* (MH^+) 393.

5.1.13. 4-((1-(2-Methoxy-2-oxoethyl)-2,2-dimethyl-3-((*R*)-1-(naphthalen-1-yl)ethylcarbamoyl)-5-oxoimidazolidin-4-yl)methyl)-1,2-phenylene diacetate (16)

A solution of **15a** or **15** (0.05 g, 0.12 mmol) and (*R*)-1-(1-naphthyl)-ethyl isocyanate (0.018 mL, 0.10 mmol) in chloroform (5 mL) was stirred overnight at rt. The solvent was removed under reduced pressure to give a residue that was purified by column chromatography using a mixture of ethyl acetate/hexane 8:2. After evaporation of the solvent the product was obtained as white solid.

5.1.13.1. Data for 4-(((*S*)-1-(2-methoxy-2-oxoethyl)-2,2-dimethyl-3-((*R*)-1-(naphthalen-1-yl)ethylcarbamoyl)-5-oxoimidazolidin-4-yl)methyl)-1,2-phenylene diacetate (16a). Mp 115–117 °C. $[\alpha]_D^{20} = +48.0$ (*c* 1, MeOH); ^{1}H NMR (DMSO-d_6): δ 8.30 (d, 1H, ArH); 7.94–7.49 (m, 6H, ArH); 6.93–6.81 (m, 2H, ArH); 6.52–6.42 (m, 2H, ArH and NH); 5.81 (m, 1H, CH); 4.72 (d, 1H, CH); 3.89 (s, 2H, CH_2); 3.62 (s, 3H, OCH_3); 3.06–2.81 (m, 2H, CH_2); 2.19 (s, 6H, CH_3); 1.59 (d, 3H, CH_3); 1.45 (s, 3H, CH_3); 0.69 (s, 3H, CH_3). Anal. ($C_{32}H_{35}N_3O_8$) C, H, N. ESIMS: *m*/*z* $(M-H)^-$ 588.

5.1.13.2. Data for 16 as a mixture of its diastereoisomers. ^{1}H NMR (DMSO-d_6): δ 8.30–8.15 (m, 1H, ArH); 7.99–7.42 (m, 6H, ArH); 7.20–6.81 (m, 3H, ArH and NH); 6.55–6.48 (m, 1H, ArH); 5.86–5.65 (m, 1H, CH); 4.82–4.73 (dd, 1H, CH); 3.92 and 3.90 (2s, 2H, CH_2); 3.62 (s, 3H, OCH_3); 3.10–2.80 (m, 2H, CH_2); 2.22 and 2.19 (2s, 6H, CH_3); 1.62–1.57 (m, 3H, CH_3); 1.45 and 1.35 (2s, 3H, CH_3); 0.75 and 0.69 (2s, 3H, CH_3). Anal. ($C_{32}H_{35}N_3O_8$) C, H, N. ESIMS: *m*/*z* $(M-H)^-$ 588.

5.2. HPLC–UV assays

All analyses were carried out 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 X-Terra RP_{18} (5 μm, 3.0 × 15 mm); the mobile phase was a mixture of water/methanol (60:40). The flow rate was 1.0 mL/min.

5.2.1. Aqueous solubility

The aqueous solubility of prodrugs was determined in deionized water.

An excess of compound was added to water and the suspensions were shaken for 15 min then filtered (Millipore 0.45 μm). The filtered solutions were analyzed by HPLC.

5.2.2. Octanol/water partition coefficients (log *P*)

Octanol/water partition coefficients were determined by placing approximately 5 mg of compound in 1 mL of anhydrous *n*-octanol, shaking vigorously for about 2 min then 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.

5.2.3. Kinetics of hydrolysis in aqueous solutions

A 0.02 M hydrochloric acid buffer of pH 1.3 as nonenzymatic 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 the respective prodrug to 10 mL of the appropriate thermostated (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 prodrugs were then calculated from the slopes of the linear plots of log (% residual prodrugs) against time. The experiments were run in triplicate for each prodrug and the mean values of the rate constants were calculated.

5.2.4. Kinetics of hydrolysis in plasma

Plasma from rats and humans was obtained by centrifugation of blood samples containing 0.3% citric acid at 3000 *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 5000*g*, 10 μL of the supernatant layer were analyzed by chromatography as described above. The amounts of remaining intact prodrug were plotted as a function of incubation time.

5.3. DPPH–HPLC assay

Fresh DPPH stock solution (500 μM in methanol) was prepared on each day of analysis. Stock solutions of antioxidants were prepared at concentration of 600, 300, 100, and 50 μM in methanol. An aliquot of 100 μL of different concentrations of antioxidants were added to 100 μL of DPPH stock solution (final concentration 250 μM). The mixture was vortexed for few seconds and left to stand in the dark for 20 min at room temperature.[23,24]

The blank was prepared by adding 100 μL of methanol to 100 μL of DPPH stock solution before each run. The mobile phase was a mixture of water/methanol (20:80) at a flow rate of 1.0 mL/min. The DPPH peaks were monitored at 517 nm. The difference in the reduction of DPPH peak area between the blank and the sample was used for determining the percent radical-scavenging activity of the sample.

5.4. HPLC–EC assays

The HPLC–EC system consisted of a PU-2080 Plus pump (Jasco), a Rheodyne 7295 injector with a 10 μL loop and an ESA Coulochem III detector. Separation was achieved on a Waters Symmetry RP-C_{18} column (4.6 mm × 150 mm, 5 μm).

The mobile phase consisted of 0.045 M monobasic sodium phosphate, 0.001 M 1-octanesulfonic 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.

The electrochemical detection system included a high sensitivity dual detector analytical cell: detector 1 set at +350 mV; detector 2 set at −180 mV. The signal was recorded using the response from detector 1.

5.4.1. Sample processing

For preparation of the samples, the striatal tissue from each animal 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-octanesulfonic acid sodium salt, 0.060% heptanesulfonic 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 4500*g* for 10 min and the supernatant was filtered using 0.45 μm Millipore filters. The filtrate was set in a low volume insert vial and a portion was injected directly into the liquid chromatography equipment (10 μL).[25]

5.4.2. Preparation of standards

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-octanesulfonic acid sodium salt, 0.060% heptanesulfonic acid sodium salt, 0.004% sodium EDTA, and 0.010% sodium metabisulfite. These standard solutions were freshly prepared every week and stored at 4 °C for use right away.

5.4.3. Peak identification

The monoamine and their metabolites were identified on the basis of retention time. The concentration of each compound was established from the peak area ratio using DHBA as internal standard. Final values were expressed in terms of picomoles per gram of tissue. Measurements were performed in triplicate for each original sample. Results are expressed as mean ± standard deviation (SD).

5.4.4. Chemicals and reagents

The DA, DOPAC, DHBA, 1-octanesulfonic acid sodium salt, sodium metabisulfite, and acetonitrile were purchased from Sigma; *o*-phosphoric acid, perchloric acid, heptanesulfonic acid sodium salt, and sodium EDTA were obtained from Fluka.

Deionized water (Milli Q-Plus system, Millipore) was used for the preparation of all solutions.

5.4.5. Pharmacological treatment

Benserazide hydrochloride, a peripheral dopa-decarboxylase inhibitor, L-DOPA, **6a** and **6b** prodrugs were dissolved in dimethyl sulfoxide. All animals received a dose of benserazide (16.36 mg/kg) combined with L-DOPA (65.46 mg/kg), **6a** (102.37 mg/kg) or **6b** (111.67 mg/kg) in equimolar doses (0.332 mmol/kg). The drugs were given at a volume of 5 mL/kg in a single ip administration. A control group (n = 20), receiving only DMSO (5 mL/kg), was included in the experiment. 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).

1, 2, 3, 4, 5, and 6 h after treatment with drugs, three animals from each group (L-DOPA-treated, n = 18; **6a**-treated, n = 18; **6b**-treated, n = 18) were weighed and sacrificed by exposure to CO_2, and striatum were dissected out. Striatum were frozen in liquid nitrogen and stored at −80 °C until use.

Acknowledgments

University of Camerino, MIUR, and Fondazione CARIMA are gratefully acknowledged for their financial support.

References and notes

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