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Synthesis and *in vitro* studies on a potential dopamine prodrug

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Dopamine delivery to the central nervous system (CNS) undergoes the permeability limitations of blood-brain barrier (BBB) which is a selective interface that excludes most water-soluble molecules from entering the brain. Neutral amino acids permeate the BBB by specific transport systems. Condensation of dopamine with neutral amino acids could afford potential prodrugs able to interact with the BBB endogenous transporters and easily enter the brain. The synthesis and characterization of the dopamine derivative 2-amino-*N*-[2-(3,4-dihydroxy-phenyl)-ethyl]-3-phenyl-propionamide (**7**) is described. The chemical and enzymatic stability of **7** was evaluated. The molecular weight (300 Da) and Log P_{app} (0.76) indicated that the physico-chemical characteristics of compound **7** are adequate to cross biological membranes. Compound **7** was enzymatically cleaved to free dopamine in rat brain homogenate ($t_{1/2} = 460$ min). In human plasma, the $t_{1/2}$ of **7** was estimated comparable to that reported for L-DOPA. In view of a possible oral administration of **7**, studies of its chemical behavior under conditions simulating those of the gastrointestinal tract showed that no dopamine production occurred; furthermore, **7** is able to permeate through a simulated intestinal mucosal membrane. The collected data suggest that compound **7** could be considered a very valuable candidate for subsequent *in vivo* evaluation.

1. Introduction

Drug delivery to the brain is one of the most challenging fields of research and development for pharmaceutical products. The drug transfer from blood to brain is severely restricted by the blood brain barrier (BBB) as the tight junctions, sandwiched between the cerebral endothelial cells (ECs), form a selective diffusion barrier which excludes most blood-borne substances from entering (Scherrmann 2002; Ballabh et al. 2004; Lo et al. 2001; Ghersi-Egea et al. 1994; Wolburg and Lippoldt 2002; Huber et al. 2001). The rate at which molecules cross the BBB is related to their physico-chemical characteristics; lipid solubility, hydrogen bonding, charge, molecular size, ionization profile, flexibility or other parameters play an important role (Crivori et al. 2000; Abraham et al. 1994).
Dopamine is one of the most important neurotransmitters in the central nervous system (CNS) and its striatal depletion is responsible of clinical signs of Parkinson's disease (PD) (Antolin et al. 2002). Owing to the high hydrophilicity and the absence of a specific transport system within the membrane of ECs, dopamine is unable to cross the BBB, thus its use is precluded in treatment of PD. On the other hand, the dopamine precursor L-DOPA is actively transported into the brain through the LNAA carrier (Pahwa and Koller 1998). Currently, PD therapy is basically symptomatic and L-DOPA is the treatment of choice for this neurodegenerative disease (Tolosa et al. 1998). The drug uptake into the brain is followed by aromatic L-amino acid decarboxylase enzymatic conversion to dopamine (Pardridge 1977, 1995; Oldendorf et al. 1983). Unfortunately, upon continuous treatment, the clinical response to oral L-DOPA is variable and unreliable since its irregular absorption and first-pass metabolism. Following oral administration, the bioavailability of L-DOPA is estimated about 5 to 15% and less than 1% of the dose reaches the brain unchanged (Standaert and Young 1996). Even if the L-DOPA dose is reduced, and the bioavailability is enhanced by co-administration of decarboxylase inhibitors, plasma half-life is only modestly prolonged, and a reduction in the frequency of drug administration is not possible (Reynolds 1993).
An attractive, non invasive way to enhance brain bioavailability of dopamine could be the prodrug approach, which consists in covalent linking of actives with specific molecules able to shuttle the drug into CNS (Anderson 1996). Various dopamine prodrugs have been described; among them acyl- (Casagrande and Ferrari 1973; Borgman et al. 1973) glycosyl- (Fernández et al. 2000, 2003), amido- (Bodor et al. 1981) and other derivatives have been reported (Di Stefano et al. 2008). *In vivo* studies have identified specific carrier systems for the transfer of essential neutral amino acids throughout the ECs (Terasaki and Tsuji 1994). In particular, it has been described that phenylalanine is transported using the leucine system (L)

large neutral amino acid (LNAA) carrier (Egleton and Davis 1997). In the same way, amino acidic derivatives of dopamine could interact with amino acid transporters present in the BBB and, once reached the CNS, could be enzymatically cleaved releasing dopamine into the brain (Anderson 1996; Bodor and Buchwald 1999).

In this preliminary study we developed the synthesis and characterization of an amino acidic dopamine derivative obtained by covalently binding the drug to the essential neutral amino acid phenylalanine. Since the ECs are selective for amino acids of the L series (Bickel et al. 2001; Egleton and Davis 1997; Pardridge 1995), in this study was used L-phenylalanine. In order to determine the properties of the synthesized compound as prodrug, *in vitro* stability studies have been performed in plasma and rat brain extracts. Finally, in view of p.o. administration of the dopamine amino acidic derivative, the behavior in gastrointestinal environment and the aptitude to permeate through the intestinal membrane barrier were evaluated.

2. Investigations, results and discussion

The amino acidic derivative of dopamine was successfully obtained by synthesis involving three main steps (Scheme). Compound **1** was selected as a stable dopamine derivative to avoid oxygen and light instability of dopamine itself. DCC was chosen as coupling agent (Slebioda et al. 1990). The main product of the fist step was identified as 2-(boc)amino-*N*-[2-(3,4-dimethoxy-phenyl)-ethyl]-3-phenyl-propionamide (**4**). An important role in the reaction pathway is played by the volume of solvent used: high amount of CH_2Cl_2 promote solubilization of **3** which, in turn, reacts with activated **2** and originates the by-product **5** (Scheme). To compound **5** was attributed the structure of [1-benzyl-2-(1,3-dicyclohexyl-ureido)-2-oxo-ethyl]-carbamic acid *tert*-butyl ester which was confirmed by reacting compound **3** with compound **1** in the same experimental conditions. By removing of the Boc-protection group from **4** (second step), compound **6** was obtained. Demethylation of **6** (McOmie et al. 1968; Chen et al. 1998; Ragot et al. 1999) afforded the desired 2-amino-*N*-[2-(3,4-dihydroxy-phenyl)-ethyl]-3-phenyl-propionamide **(7)**. Spectral data of synthesized compounds were in agreement with the proposed structures (Tables 1 and 2).

The drug lipophilicity is an important factor conditioning brain uptake and the apparent partition coefficient (P_{app}) could be used as simple descriptor of ability to cross the BBB: values of log P_{app} within -0.2 to 1.3 have been described as optimal for cerebral transport; on the other hand higher values than these could reduce the rate of transport inside the membrane (Levin 1980; Gimenez et al. 2004). In view of a potential application of com-

Scheme

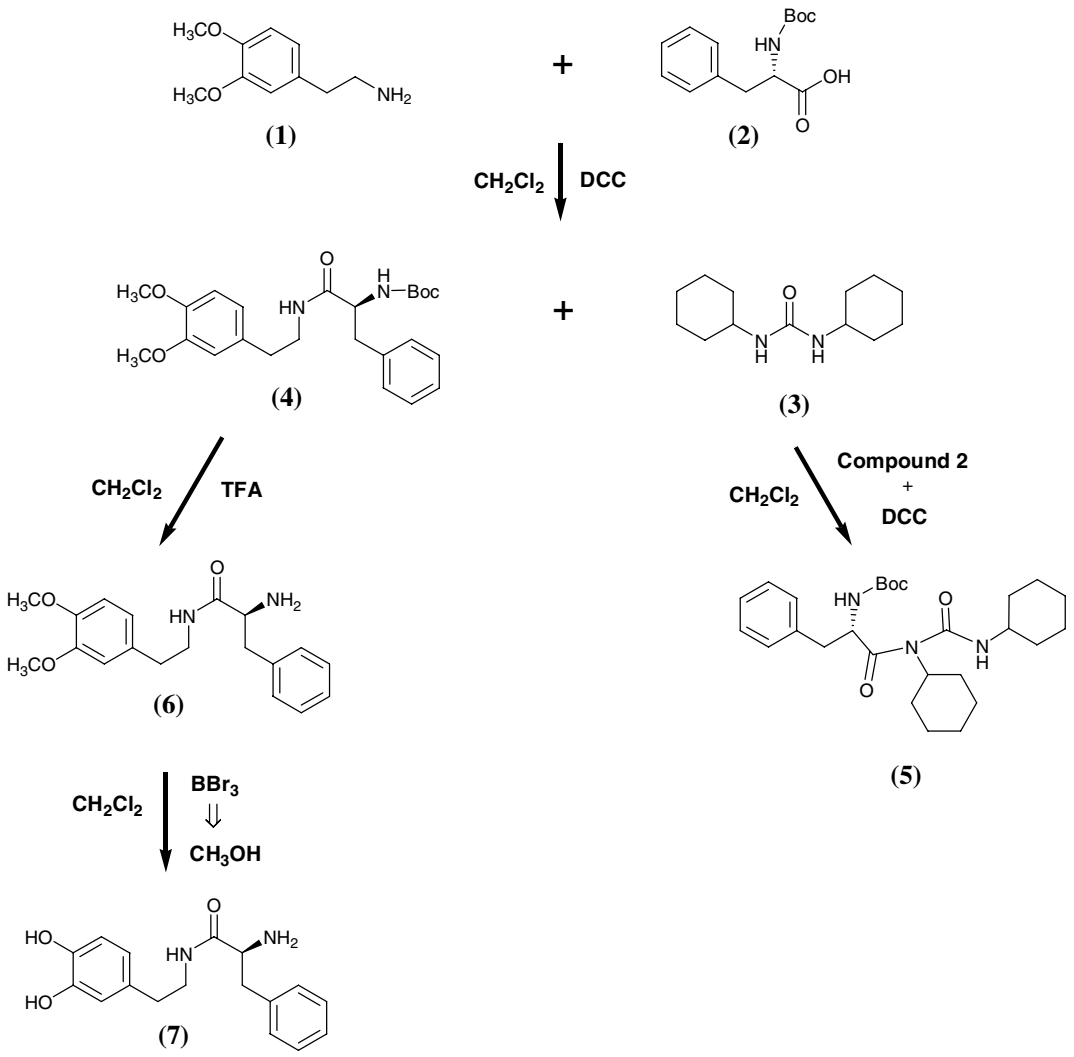


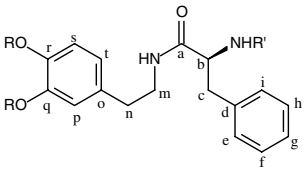
Table 1: Yield, m.p., UV, IR, MS of compounds 4, 5, 6 and 7

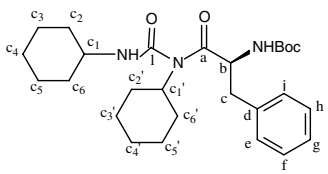
Compd.	Yield (%)	m.p. (°C)	UV (methanol) (λ_{max}, nm)	IR (cm^{-1})								MS (M^+)
				–OH	*–NH_2*	*–NH–*CO	*–NH–*CO	NH–*CO*	BOC–*CO*	*C–O–C*	N–*CO*–N	
4	50	150–152	279.4	–	–	3346	1520	1657	1682	1239, 1167		428
5	10	85–87	258.5	–	–	3306	1522	1654	1682	1226, 1170	1716	471
6	68	104–105	279.4	–	3289	3349	1510	1637	–	–	–	328
7	89	218–220	283.0	3521	3230–3100		1510	1670	–	–	–	301 $(M+1)^+$

pound **7** as prodrug, the octanol-water P_{app} was determined as preliminary parameter. Log P_{app} of compound **7** was determined as 0.76 thus suggesting that the synthesized product possesses adequate characteristics to permeate membranes.

The aptitude of compound **7** to undergo cleavage by cerebral enzymes and produce dopamine *in situ* was evaluated using rat brain homogenate. We experienced that compound **7** slowly decreased in concentrations while raising amounts of dopamine were formed (Fig. 1). Experimental data were curve-fitted to the most common kinetic equations and the best fit was obtained using the Michaelis-Menten relationship thus suggesting a typical enzymatic saturation kinetic of cleavage. By extrapolation the $t_{1/2}$ was estimated about 460 min which is considerably higher than that reported for dopamine degradation ($t_{1/2} = 180$ min) in the

Table 2: ^{13}C and ^{1}H (in parenthesis) NMR data of compounds 4, 5, 6 and 7





Compd.	**4**	**6**	**7**	Compd.	**5**
C-a	170.97	173.84	169.30	C-a	170.72
C-b	56.15 (4.24)	56.17 (3.57)	55.72 (4.08)	C-b	54.51 (4.61)
C-c	38.76 (3.01, 3.05)	40.77 (2.69, 3.22)	38.63 (3.03, 3.13)	C-c	39.06 (2.83, 3.05)
C-d	136.77	137.62	135.50	C-d	136.16
C-e, i	129.29 (7.18)	129.07 (7.20)	130.51 (7.30)	C-e, i	129.14 (7.17)
C-f, h	128.62 (7.27)	128.41 (7.30)	129.99 (7.24)	C-f, h	128.57 (7.32)
C-g	126.91 (7.26)	126.54 (7.22)	128.74 (7.31)	C-g	127.96 (7.31)
				C-l	152.91
(C-a)NH	(5.76)	(7.37)	(8.27)	(C-l)NH	(5.08)
C-m	40.68 (3.40)	40.17 (3.48)	42.25 (3.34)	C-1	50.02 (3.65)
C-n	35.11 (2.61)	35.13 (2.73)	35.52 (2.56)	C-2,6	32.38 (1.26, 1.93)
C-o	131.06	131.35	131.58	C-3,5	25.25 (1.31, 1.68)
C-p	111.80 (6.63)	111.84 (6.70)	116.78 (6.65)	C-4	25.40 (1.17, 1.60)
C-q	149.10	148.80	146.19	C-1′	54.51 (4.11)
C-r	147.76	147.46	144.77	C-2′,6′	31.28 (1.35, 1.91)
C-s	111.40 (6.76)	111.26 (6.78)	116.39 (6.68)	C-3′,5′	25.95 (1.29, 1.70)
C-t	120.55 (6.57)	120.45 (6.68)	121.03 (6.49)	C-4′	25.86 (1.13, 1.60)
R =(C-q)OCH_3	55.85 (3.85)	55.64 (3.83)			
R =(C-r)OCH_3	55.90 (3.84)	55.69 (3.83)			
R = OH			(*)		
R′= H; $-NH_2$		(4.79)	(7.26)		
R′= Boc; –NH	(5.01)			(Boc)–NH	(5.08)

R′= Boc: 155.25; 80.15; 28.23 (1.39).

Boc: 155.83; 80.20; 28.14 (1.39).

(*) Rapidly exchangeable protons

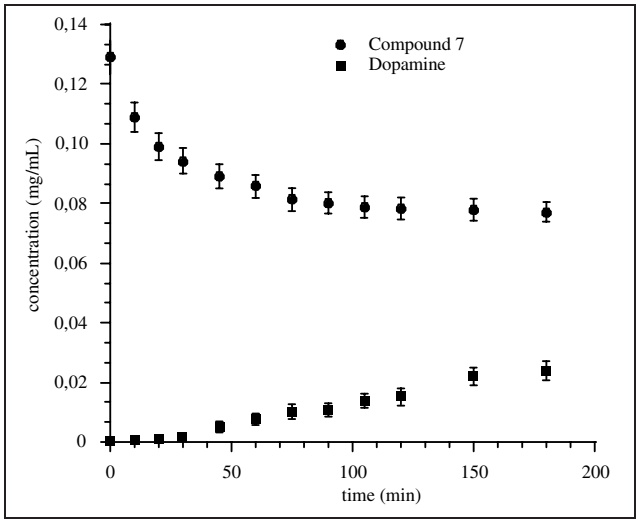


Fig. 1: Time courses of compound **7** cleavage and simultaneous formation of dopamine in rat brain homogenate at 37 ± 0.5 °C. Values are presented as means ± SD (n = 3)

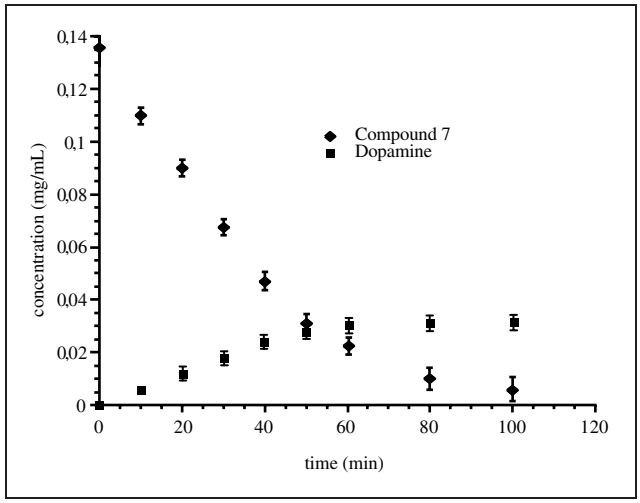


Fig. 2: Profiles of disappearance of compound **7** and simultaneous formation of dopamine in human plasma at 37 ± 0.5 °C. Values are presented as means ± SD (n = 3)

same experimental conditions (Fernandez et al. 2000). This result suggests that compound **7** could be considered as a reservoir of dopamine into the CNS.

Stability was evaluated in plasmatic environment following the *in vitro* disappearance of **7** from human plasma and simultaneous appearance of dopamine (Fig. 2). As above, the hydrolysis occurred following typical enzymatic saturation kinetics. By extrapolation, the $t_{1/2}$ in human plasma was estimated about 28 min. It is noteworthy that this value is comparable to that reported for L-DOPA (Gennaro 2000).

In view of a possible oral administration of compound **7**, studies of chemical stability of **7** in conditions simulating those of gastrointestinal tract (37 °C, pH 1.2 to 8.0) were performed. Experimental data showed that the peptide bond of **7** remains uncleaved and no production of dopamine was observed. UV measurements experienced that absorption peak of aqueous solution of **7** occurred at $\lambda = 281.0$ nm, no shifts in the pH range 1.2 to 6.5 were observed. This behavior remained unchanged up to 6 h, thus indicating high stability at pH conditions of gastrojejunum tract (Fig. 3a and b). For solutions at pH > 7, after about 15 min, the appearance of a broad peak centered at $\lambda = 458.5$ nm was observed which increased with time suggesting a degradation process (Fig. 3c and d). The degradation trend was followed graphically reporting the absorbance values at $\lambda = 458.5$ nm vs time (Fig. 4). The rate of formation of the species that absorbs at $\lambda = 458.5$ nm increased with pH, indicating a base-catalyzed process. Likewise the most dihydroquinone derivatives, dopamine in alkaline media is easily auto-oxidized in presence of molecular oxygen. During this chain process, dopamine is transformed into a semiquinone species by hydroxyl ions.

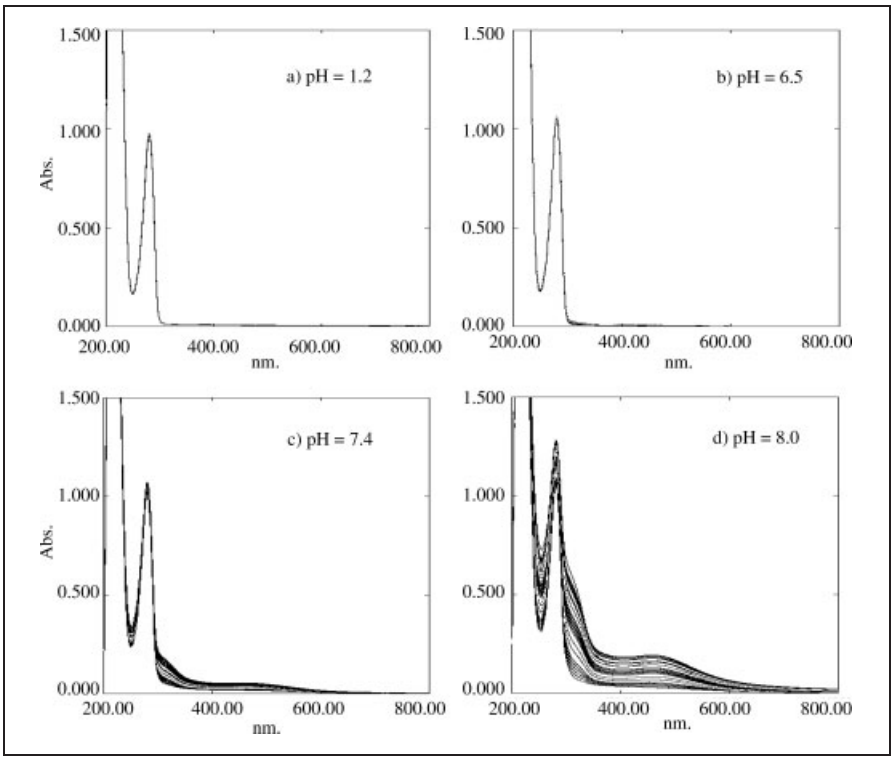


Fig. 3:
UV measurements of aqueous solution of **7**, scanned every 15 min up to 360 min, at a) pH 1.2, b) pH 6.5, c) pH 7.4, d) pH 8.0. An increase in absorbance was detected for solutions at pH > 7 at $\lambda = 280.5$ and $\lambda = 458.5$: it was 0.0020 ± 0.0003 and 0.0015 ± 0.0005 respectively for solutions at pH 7.4 whereas the increase was 0.0080 ± 0.0001 and 0.0050 ± 0.0002 respectively for solutions at pH 8

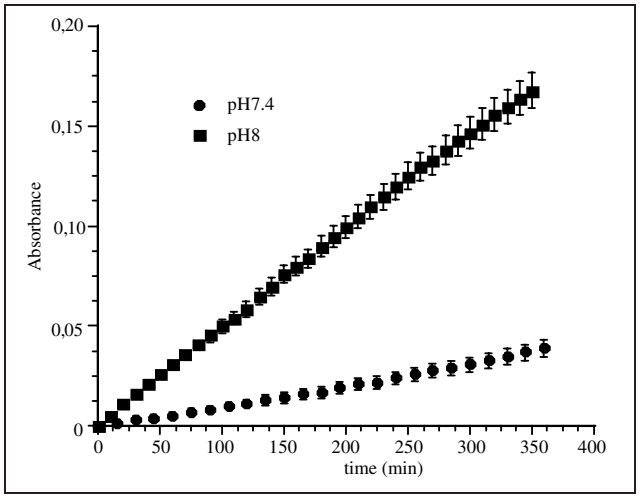


Fig. 4: Time courses of absorbance of peak at $\lambda = 458.5$ nm of aqueous solutions of **7** at pH 7.4 and 8.0. Values are presented as means $\pm$ SD (n = 5)

The semiquinone derivative is oxidized to an open-chain quinone which, in turn, undergoes intramolecular-pH dependent cyclization to dopaminochrome and others byproducts. This very fast degradation pathway has been described on the basis of UV spectrophotometric studies (Klegeris et al. 1995; Terland et al. 1997). In analogy, in our experiments, the appearance of the peak at $\lambda = 458.5$ nm at pH > 7 is indicative of the same base-catalysed autoxidation mechanism for compound **7**.

In p.o. drug administration, a further limiting step to bioavailability could be permeation across the gastro-enteric mucosal tissue. The P_{app} value of compound **7** suggested that it has suitable characteristics to cross the intestinal membrane (Scherrmann 2002; Balimane et al. 2000). In this study, the aptitude of compound **7** to permeate through a membrane simulating the intestinal barrier was evaluated (Zhu et al. 2002). Diffusion of **7** through the membrane was reported as cumulative amount of permeated **7** per unit area vs time (Fig. 5). At the steady state the flux (J_s) and the permeability coefficient (K_p) do not change significantly (t-test) with the pH and resulted 20.08 ± 1.71 μg/cm^2 h and 0.10 ± 0.01 cm/h respectively.

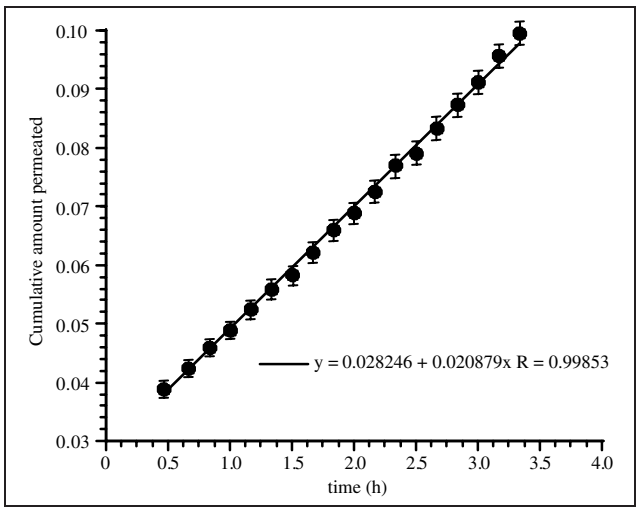


Fig. 5: Cumulative amount (mg) of compound **7** permeated across simulated duodenum/jejunum/ileum barrier per unit area *versus* time. Values are presented as means $\pm$ SD (n = 5)

These preliminary data suggest that **7** could be able to cross membranes of the duodenum/jejunum/ileum tract.

The collected data suggest that compound **7** fulfills the prodrug criteria and could be considered a very valuable candidate for subsequent evaluation *in vivo*.

3. Experimental

3.1. Materials and apparatus

Trifluoroacetic acid (TFA), 2-(3,4-dimethoxyphenyl)-ethylamine (**1**), egg lecithin (phosphatidylcoline), *n*-dodecane and all components of buffer solutions were purchased from Sigma-Aldrich-Chemie (Steinheim, Germany). *N*-Boc-L-phenylalanine (**2**), *N,N′*-dicyclohexylcarbodiimide (DCC) and 1M CH_2Cl_2 solution of boron tribromide (BBr_3) were from Acros (Milan, Italy). HPLC grade solvents were obtained from Baker (Milan, Italy). All other chemicals and solvents were of analytical grade and were used without further purification. The process of reactions was monitored by TLC using Alugram® SIL G/UV$_{254}$ (Macherey-Nagel) pre-coated aluminium sheets (0.20 mm layer thickness) and visualised using an UV lamp. Melting points (m.p.) were determined with a Büchi 530 capillary apparatus and are uncorrected. IR spectra were recorded with a Perkin-Elmer 1720 FT spectrophotometer as nujol mulls and UV spectra with a Shimadzu UV-Vis 1700 PharmaSpec spectrophotometer. In chemical and enzymatic stability tests, HPLC analyses were performed with a Shimadzu LC-10AD$_{VP}$ instrument equipped with a binary pump LC-10AD$_{VP}$, a UV SPD-M20A Diode Array detector, a 20 μl injector and a computer integrating apparatus (EZ Start 7.3 software). Chromatographic separation was achieved on a reversed-phase column Discovery HS-F5 (Supelco, 5 μm, 25 cm × 4.6 mm), mobile phase consisted of a mixture of 0.01% TFA in distilled water and acetonitrile (gradient profile: 95 : 5 → 93 : 7 → 70 : 30 → 95 : 5). The flow rate was set at 1 mL/min and the UV wavelength at 280.5 nm. In these conditions, the retention times for dopamine and compound **7** were 11 min and 34 min, respectively. Standard curves were used for quantification of integrated areas under the peaks. The calibration curves were performed at the concentration range of 2 to 20 mg/100 mL. In these conditions the detection limits (LOD) were < 0.002 mg/mL. ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ solutions on a Bruker AC 250 spectrometer operating in FT mode at 250.13 and 62.89 MHz, respectively. Only for compound **7** spectra were recorded in CD_3OD solution as it was very scarcely soluble in $CDCl_3$. ^{1}H and ^{13}C NMR chemical shift values were measured relative to $CDCl_3$ centered respectively at 7.25 and 77.00 ppm downfield from TMS or relative to CD_3OD centered respectively at 3.31 and 49.00 ppm downfield from TMS. ^{13}C NMR chemical shift values were determined from proton fully decoupled spectra with the assignment supported by 1D and 2D experiments performed using the standard Bruker pulse sequences DEPT.AUR, XHDEPT.AUR and COLOC.AUR. Electrospray ionization-mass (ESI-MS) spectrometry was recorder on a Autospec Tof Ultima, MicroMass Magnetic Sector Orthogonal Tof Spectrometer (spray voltage 4000 V; cone voltage 20 V, capillary temperature 40 °C, collision gas: argon 1×10^{-6} atm). Experimental data were elaborated using Kaleidagraph and Curve Expert 1.34 for Windows software.

3.2. Synthetic pathway

3.2.1. Synthesis of 2-(boc)amino-N-[2-(3,4-dimethoxy-phenyl)-ethyl]-3-phenyl-propionamide (**4**)

To a dry CH_2Cl_2 solution (10 mL) of **1** (0.92 g, 0.005 mol) and **2** (1.33 g, 0.005 mol), DCC (1.25 g, 0.005 mol) was added as coupling agent. The mixture was maintained at 0 °C in an ice bath with constant stirring for 3 h. The reaction was monitored by TLC using petroleum ether/chloroform/ethyl acetate = 10/65/25 as eluent. The mixture was then filtered to separate the solid *N,N′*-dicyclohexylurea **(3)** formed from DCC. The filtered solution was evaporated under reduced pressure. The resulting oily mass was treated with anh. ice-cooled acetone (20 mL) to separate additional **3**. The acetone solution was evaporated under reduced pressure and the crude resulting mass was purified by crystallization from cyclohexane followed by recrystallization from ethanol/water (1/5). Two recrystallizations were sufficient to obtain a pure sample of a product identified as 2-(boc)amino-*N*-[2-(3,4-dimethoxy-phenyl)-ethyl]-3-phenyl-propionamide (**4**). Yield, m.p. and spectral data are given in Tables 1 and 2. MS: m/z (relative abundance %) 428 (16, M^+), 164 (100, $(CH_3O)_2C_6H_3$-CH_2-CH_2^+), 151 (20, $(CH_3O)_2C_6H_3$-CH_2^+), 120 (24, C_6H_5-CH_2-CH-NH_2^+), 104 (4, C_6H_5-CH_2-CH^+), 91 (23, C_6H_5-CH_2^+), 77 (5, $C_6H_5^+$), 57 (13, ter-butyl$^+$).

From cyclohexane mother liquors, after evaporation, a by-product was separated which was recrystallized twice from ethyl acetate/petroleum ether (1/3) and identified as [1-benzyl-2-(1,3-dicyclohexyl-ureido)-2-oxo-ethyl]-carbamic acid *tert*-butyl ester (**5**).

Yield, m.p. and spectral data are given in Table 1 and 2.

3.2.2. Hydrolysis of compound **4** *to 2-amino-N-[2-(3,4-dimethoxy-phenyl)-ethyl]-3-phenyl-propionamide* (**6**)

To a solution of TFA (5 mL) in CH_2Cl_2 (10 mL) were added 400 mg (0.0009 mol) of compound **4.** The mixture was allowed to react in an ice bath for 4 h with constant stirring. The reaction was monitored by TLC using ethyl acetate/methanol/chloroform = 50/20/30 as eluent. At the end of the reaction, to the mixture was added dropwise a saturated water solution of $NaHCO_3$ until pH 8 was reached. The organic phase was separated and treated with solid anh. Na_2SO_4 to eliminate water residues, filtered and evaporated under reduced pressure. The residual solid mass was purified by crystallization from cyclohexane. Two recrystallizations were sufficient to obtain a pure sample of a product identified as 2-amino-*N*-[2-(3,4-dimethoxy-phenyl)-ethyl]-3-phenyl-propionamide (**6**). Yield, m.p. and spectral data are given in Tables 1 and 2.

3.2.3. Demethylation of compound **6** *to 2-amino-N-[2-(3,4-dihydroxy-phenyl)-ethyl]-3-phenyl-propionamide* (**7**)

To a dry CH_2Cl_2 (20 mL) solution of **6** (100 mg, 0.00030 mol) BBr_3 (1.8 mL, 0.00180 mol) was added and the mixture was stirred for 2 h at room temperature. Methanol (20 mL) was then added and the reaction set aside at room temperature for further 2 h under constant stirring. The reaction was monitored by TLC using a mixture of chloroform/methanol = 80/20 as eluent. At the end of the reaction, the mixture was evaporated under reduced pressure. To the crude mass was added excess methanol and the solution was evaporated off under reduced pressure, to eliminate trimethylborate formed. The residue was then heated to eliminate methyl bromide and hydrobromic acid. The oily mass obtained was washed with ethyl acetate (10 mL × 5) to obtain a solid product. The TLC showed only one chromatographic spot. To the product was assigned the structure of 2-amino-*N*-[2-(3,4-dihydroxy-phenyl)-ethyl]-3-phenyl-propionamide (**7**) which was obtained as hydrobromide salt. Yield, m.p. and spectral data are given in Tables 1 and 2. MS: m/z (relative abundance %) 301 (100, $M + 1^+$), 137 (22, $(HO)_2C_6H_3\text{-}CH_2\text{-}CH_2^+$), 123 (4, $(HO)_2C_6H_3\text{-}CH_2^+$), 120 (76, $C_6H_5\text{-}CH_2\text{-}CH\text{-}NH_2^+$), 104 (11, $C_6H_5\text{-}CH_2\text{-}CH^+$), 91 (16, $C_6H_5\text{-}CH_2^+$), 77 (6, $C_6H_5^+$).

3.3. Determination of apparent partition coefficient (P_{app}) of compound 7

Apparent partition coefficient (P_{app}) of compound **7** (2.5 mg) was determined, at 20 °C and at pressure of 1.013×10^5 Pa, in *n*-octanol (20 mL)/0.02 M phosphate buffer pH 7.4 solution (20 mL) and expressed as Log P_{app}. Following 5 min shaking at time intervals of 10 min for 3 h in a separator funnel, the mixture reached equilibrium. After partition, the organic phase was separated from the aqueous one, dried with anh. solid Na_2SO_4 and filtered. The concentration of **7** in the octanol and aqueous layers was determined by UV spectrophotometric analysis using the appropriate calibration curve and blank. The calibration curves were performed at the concentration range 2 to 12 mg/100 mL in both media. P_{app} was calculated according to equation:

$$P_{app} = \frac{C_i - C_w}{C_w}\left(\frac{V_w}{V_o}\right)$$

where C_i and C_w represent the drug concentration before and after partition, respectively. V_w and V_o are the volumes of the aqueous and the oil phase. The experiments were repeated five times.

3.4. Stability of compound 7

3.4.1. Stability in rat brain homogenate

Sprague-Dawley rats (Stefano Morini, San Polo d'Enza, Italy) weighing 300–350 g were housed in our institutional animal care facility under direction of a licensed veterinary. Procedures were conducted according to local and international guidelines on the ethical use of animals. Rats were sacrificed by decapitation and the brains were immediately removed, weighed and homogenized in buffer solution (nuclei buffer containing: 50 mM Na_2HPO_4, 50 mM NaH_2PO_4, 50 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 2 mM EDTA, 0.5 mM EGTA, 0.32 M sucrose). The homogenate was centrifuged at 60,000 × g for 40 min at 0 °C and the surnatant was subdivided in aliquots of 400 µl which were stored at −20 °C until used. Experiments started by adding 50 µl of stock solution of compound **7** (2.075 mg/mL in phosphate buffer pH 6.5 solution) to a 400 µl aliquot of brain homogenate previously equilibrated at 37 ± 0.5 °C. At appropriate time intervals, samples (25 µl) were withdrawn and, after quenching the reaction by adding 20 µl of acetonitrile, were analyzed by HPLC as above described. The experiments were carried out for 3 h and repeated three times.

3.4.2. Stability in human plasma

The stability of compound **7** was investigated in 80% human plasma diluted in PBS pH 7.4. The reaction was initiated by adding 100 µl of stock solution of **7** (2.05 mg/mL in isotonic buffer pH 7.4) to 800 µl of preheated (37 ± 0.5 °C) plasma. The mixture was kept in a water bath at 37 ± 0.5 °C. At appropriate time intervals samples (25 µl) were taken, quenched by adding the same volume of acetonitrile, and centrifuged (15,000 × g, 10 min). The supernatant was analysed by HPLC as above described. The experiments were carried out for 2 h and repeated three times.

3.4.3. Stability in buffer solutions simulating biological fluids

Chemical stability of compound **7** was studied at 37 ± 0.5 °C in 50 mM sodium citrate/HCl (pH 1.2), phosphate (pH 6.5, 7.4, 8.0) buffers adjusted to an ionic strength of µ = 0.5 by addition of a calculated amount of sodium chloride. Experiments were initiated by adding 15 mg of compound **7** in 100 mL of the appropriate buffer solution. In all experiments, to evaluate the peptide bond stability, an aliquot of solution was kept in a water bath and every 15 min samples (50 µl) were withdrawn and immediately analysed by HPLC, as above described. Moreover, to assess potential molecular rearrangements, a further aliquot of the solution (3 mL) was placed in a quartz UV cell, maintained at constant temperature, and scanned every 15 min (λ ranging from 200 to 800 nm). The experiments were carried out for 6 h and repeated five times.

3.5. In vitro permeation of compound 7 *through artificial intestinal barrier*

A solution of compound **7** (0.2 mg/mL in phosphate buffer pH 5.0, 6.0 and 7.0 solutions simulating duodenum/jejunum/ileum juices respectively) was placed in the donor compartment of a horizontal Franz-type diffusion cell. In the acceptor compartment was placed phosphate buffer pH 7.4 solution simulating plasma. As artificial intestinal barrier, a filtration nitrate cellulose membrane (Whatman, International Ltd. Maidstone, England), wetted with egg lecithin dispersed in *n*-dodecane (1% w/v), was used (Zhu et al. 2002). At regular time intervals (10 min), samples were withdrawn from the acceptor compartment. To avoid saturation phenomena and maintain the "sink" conditions, the sample volume taken out was replaced by fresh fluid. Transfer of **7** from the donor to the acceptor compartment was monitored by HPLC measurements of the amount of **7** that reached the acceptor fluid. The experiments were carried out for 4 h and repeated five times. The flux value (J_s) and the permeability coefficient (K_p) were calculated at the steady state per unit area by linear regression analysis of permeation data (Giannola et al. 2007).

3.6. Statistical analysis

The statistical significance was determined by Student's t-test. A probability level of 0.05 or smaller was used to indicate statistical significance.

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References

Abraham MH, Chada S, Mitchell R (1994) Hydrogen bonding. 33 Factors that influence the distribution of solutes between blood and brain. J Pharm Sci 83: 1257–1268.

Anderson BD (1996) Prodrugs for improved CNS delivery. Adv Drug Deliv Rev 19: 171–202.

Antolin I, Mayo JC, Sainz RM, de los Angeles del Brio M, Herrera F, Martin V, Rodriguez C (2002) Protective effect of melatonin in a chronic experimental model of Parkinson's disease. Brain Res 943: 163–173.

Balimane PV, Chong S, Morrison RA (2000) Current methodologies used for evaluation of intestinal permeability and absorption. J Pharmacol Toxicol Meth 44: 301–312.

Ballabh P, Braun A, Nedergaard M (2004) The blood-brain barrier: an overview. Structure, regulation, and clinical implications. Neurobiol Disease 16: 1–13.

Bickel U, Yoshikawa T, Pardridge WM (2001) Delivery of peptides and proteins through the blood-brain barrier. Adv Drug Deliv Rev 46: 247–279.

Bodor N, Farag HH, Brewster ME (1981) Site-specific, sustained release of drugs to the brain. Science 214: 1370–1372.

Bodor N, Buchwald P (1999) Recent advances in the brain targeting of neuropharmaceuticals by chemical delivery systems. Adv Drug Deliv Rev 3: 229–254.

Borgman RJ, McPhillips JJ, Stitzel RE, Goodman IJ (1973) Synthesis and pharmacology of centrally acting dopamine derivatives and analogs in relation to Parkinson's disease. J Med Chem 16: 630–633.

Casagrande C, Ferrari G (1973) 3,4–O-Diacyl derivatives of dopamine. Farmaco 28: 143–148.

Chen HJC, Applewhite T, Jayachandran B, Kirk KL (1998) Synthesis of 4,6-difluoro-5-hydroxy-(α-methyl)tryptamine and 4,6-difluoro-5-hydroxy-(β-methyl)tryptamine as potential selective monoamine oxidase B inhibitor. J Fluorine Chem 92: 41–44.

Crivori P, Cruciani G, Carrupt PA, Testa B (2000) Predicting blood-brain barrier permeation from three-dimensional molecular structure. J Med Chem 43: 2204–2216.

Di Stefano A, Sozio P, Cerasa LS (2008) Antipakinson prodrugs. Molecules 13: 46–68.

Egleton RD, Davis TP (1997) Bioavailability and transport of peptides and peptide drugs into the brain. Peptides 18: 1431–1439.

Fernàndez C, Nieto O, Rivas E, Montenegro G, Fontenla JA, Fernàndez-Mayoralas A (2000) Synthesis and biological studies of glycosyl dopamine derivatives as potential antiparkinsonian agents. Carbohydr Res 327: 353–365.

Fernández C, Nieto O, Fontenla JA, Rivas E, de Ceballos ML, Fernández-Mayoralas A (2003) Synthesis of glycosyl derivatives as dopamine prodrugs: interaction with glucose carrier GLUT-1. Org Biomol Chem 1: 767–771.

Ghersi-Egea JF, Leninger-Muller B, Suleman G, Siest G, Minn A (1994) Localization of drug-metabolizing enzyme activities to blood-brain interfaces and circumventricular organs. J Neurochem 62: 1089–1096.

Giannola LI, De Caro V, Giandalia G, Siragusa MG, Campisi G, Florena AM, Ciach T (2007) Diffusion of naltrexone across reconstituted human oral epithelium and histomorphological features. Eur J Pharm Biopharm 65: 238–246.

Gimenez F, Fernandez C, Mabondzo A (2004) Transport of HIV protease inhibitors through the blood-brain barrier and interactions with the efflux proteins, P-glycoprotein and multidrug resistance proteins. J Acquir Immune Defic Syndr 36: 649–658.

Huber JD, Egleton RD, Davis TP (2001) Molecular physiology and pathophysiology of tight junctions in the blood-brain barrier. Trends Neurosci 24: 719–725.

Klegeris A, Korkina LG, Greenfield SA (1995) Autoxidation of dopamine: a comparison of luminescent and spectrophotometric detection in basic solution. Free Radic Biol Medic 18: 215–222.

Levin VA (1980) Relationship of octanol/water partition coefficient and molecular weight to rat brain capillary permeability. J Med Chem 23: 682–684.

Lo EH, Singhal AB, Torchilin VP, Abbott NJ (2001) Drug delivery to damage brain. Brain Res Rev 38: 140–148.

McOmie JFW, Watts ML, West DE (1968) Demethylation of aryl methyl ethers by boron tribromide. Tetrahedron Lett 24: 2287–2292.

Oldendorf WH, Crane PD, Braun LD, Wade LA, Diamond JM (1983) Blood-Brain Barrier transport of basic amino acids is selectively inhibited at low pH. J Neurochem 40: 797–800.

Pahwa R, Koller WC (1998) Advances in the treatment of Parkinson's disease. Drugs Today 34: 95–105.

Pardridge WM (1977) Kinetics of competitive inhibition of a neutral amino acid transport across the blood-brain barrier. J Neurochem 28: 103–108.

Pardridge WM (1995) Transport of small molecules through the blood-brain barrier: biology and methodology. Adv Drug Deliv Rev 15: 5–36.

Ragot JP, Steeneck C, Alcaraz ML, Taylor RJK (1999) The synthesis of 1,8-dihydroxtnaphtalene-derived natural products: palmarumycin CP1, palmarumycin CP2, palmarumycin C11, CJ-12,371, deoxypreussomerin A and novel analogues. J Chem Soc, Perkin Trans I, 1073–1082.

Reynolds JEF (1993) Martindale, The Extra Pharmacopoeia, 30th Ed, Pharmaceutical Press, London, UK, p. 844.

Scherrmann JM (2002) Drug delivery to brain via the blood-brain barrier. Vasc Pharmacol 38: 349–354.

Slebioda M, Wodecki Z, Kolodziejczyk AM (1990) Formation of optically pure N-acyl-N,N′-dicyclohexylurea in N,N′-dicyclohexyl-carbodiimide-mediated peptide synthesis. Int J Pept Protein Res 35: 539–541.

Standaert DG, Young AB (1996) Treatment of central nervous system degenerative disorders. In: Hardman JG, Limbird LE, Molinoff PB, Ruddon RW, Gilman AG (eds) Goodman & Gilman's The Pharmacological Basis of Therapeutics, 9th ed., McGraw-Hill Companies, New York, p. 503–519

Terasaki T, Tsuji A (1994) Drug delivery to the brain utilizing blood-brain barrier transport systems. J Control Release 29: 163–169.

Terland O, Flatmark T, Tangerås A, Gronberg M (1997) Dopamine oxidation generates an oxidative stress mediated by dopamine semiquinone and unrelated to reactive oxygen species. J Mol Cell Cardiol 29: 1731–1738.

Tolosa E, Marti MJ, Valldeoriola F, Molinuevo JL (1998) History of levodopa and dopamine agonists in Parkinson's disease treatment. Neurology 50: S2–S10.

White HS (2000) Skeletal muscle relaxants. In: Gennaro AR (ed.) Remington: The Science and Practice of Pharmacy, 20th Ed., Lippincott Williams and Wilkins, Philadelphia, p. 1341–1342.

Wolburg H, Lippoldt A (2002) Tight junctions of the blood-brain barrier: development, composition and regulation. Vasc Pharmacol 38: 323–337.

Zhu C, Jiang L, Chen TM, Hwang KK (2002) A comparative study of artificial membrane permeability assay for high throughput profiling of drug absorption potential. Eur J Med Chem 37: 399–407.