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# Oxazepam-dopamine conjugates increase dopamine delivery into striatum of intact rats

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## ABSTRACT

The neurotransmitter dopamine (DA) was covalently linked to oxazepam (OXA), a well-known positive allosteric modulator of γ-aminobutyric acid type-A ($GABA_A$) receptor, through a carbamate linkage (**4**) or a succinic spacer (**6**). These conjugates were synthesized with the aim of improving the delivery of DA into the brain and enhancing GABAergic transmission, which may be useful for the long-term treatment of Parkinson disease (PD). Structure-based permeability properties, *in vitro* stability and blood-brain barrier (BBB) permeability studies led to identify the OXA-DA carbamate conjugate **4a** as the compound better combining sufficient stability and ability to cross BBB. Finally, *in vivo* microdialysis experiments in freely-moving rats demonstrated that **4a** (20 mg/kg, i.p.) significantly increases extracellular DA levels into striatum, with a peak (more than 15-fold increase over the baseline) at about 80 min after a single administration. The stability and delivery data proved that **4a** may be a promising candidate for further pharmacological studies in animal models of PD.

*Keywords*:

Blood Brain Barrier

Prodrugs

Codrugs

Dopamine

GABA transmission

Parkinson disease.

*Abbreviations*: ANOVA, analysis of variance; BBB, blood-brain barrier; CDI, 1,1-carbonyldiimidazole; CNS, central nervous system; DA, dopamine; DMEM, Dulbecco's modified eagle's medium – high glucose; DMSO, dimethylsulfoxide; EDC, N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride; EDTA, Ethylenediaminetetraacetic acid; ESI, electrospray ionization mass spectrometry; FD4, Fluorescein isothiocyanate-dextran; FT-IR, Fourier transform infrared spectroscopy; GABA, γ-aminobutyric acid; $^{1}$H-NMR, Proton nuclear magnetic resonance; HPLC, high-performance liquid chromatography; i.p., intraperitoneal; LAT1, neutral amino-acid transporter 1; LC–MS, LC–mass spectrometry; LD, L-Dopa; MDCKII-MDR1, Madin–Darby canine kidney cells retrovirally transfected with the human MDR1 cDNA; PD, Parkinson's disease; S.E.M., Standard Error of Mean; tPSA, topological Polar surface area; TEA, triethylamine; TEER, trans-epithelial electrical resistance; TFA, Trifluoroacetic acid; THF, tetrahydrofurane; TLC, thin layer chromatography.

## 1. Introduction

The neurotransmitter dopamine (DA), like many other CNS active molecules, does not cross the blood-brain barrier (BBB) to enter the central nervous system (CNS), because of its hydrophilicity and the absence of endogenous transport systems through the brain capillary endothelium. Only small molecules with moderate lipophilicity and low molecular mass are able to cross the BBB via passive transport, if not substrates for the ATP-dependent drug efflux pumps.[1-3] Various approaches have been pursued to increase the brain delivery of therapeutic small molecules[4-6], including the development of prodrugs.[7,8] It has been well established that a CNS-targeted prodrug or codrug should possess either optimal physicochemical properties (e.g., lipophilicity), which allow passive diffusion through the BBB *via* the transcellular route, or structural features to serve as a substrate for one of the endogenous influx transport systems of the BBB.[9]

In this context, L-Dopa (LD), the most effective therapy for the treatment of Parkinson disease (PD)[10], is considered a prodrug of DA. LD crosses the BBB through the type 1 large neutral amino-acid transporter (LAT1) and, once in the brain, is rapidly converted to DA by aromatic amino-acid decarboxylase.[11] Although LD improves PD symptoms in the initial stages of the disease, clinical and preclinical studies have demonstrated that its long-term use is limited by the development of severe side effects, including abnormal involuntary movements (dyskinesia) and psychiatric complications.[12,13]

In PD an altered activity of the GABAergic neurotransmission and a dominance of the excitatory transmission in the basal ganglia and substantia nigra, may determine the loss of dopaminergic neurons in the pars compacta of the substantia nigra.[14] To overcome this drawback, it has been suggested that drugs enhancing γ-aminobutyric acid

(GABA) transmission could be a useful therapy for PD.[15] Indeed, it has been shown that $GABA_A$ receptors contribute to the modulation of the activity of mesocortical and mesolimbic dopaminergic neurons and to the neuroprotection of dopaminergic cells.[16] To this regard, selective GABAergic agonists, such as zolpidem, that acts within the basal ganglia, and muscimol, that decreases the output of the basal ganglia to thalamus, could be beneficial in patients who experience complications associated with long-term LD or dopaminomimetic treatments[15,16], and may also potentially be a useful treatment to modulate GABAergic tone in the basal ganglia.[17]

Recently, we have studied novel codrugs with GABAergic activity for DA delivery in the brain.[18] In this study, we investigated new conjugates obtained by linking DA and oxazepam (OXA), a well-known benzodiazepine GABA-agonist.[19] OXA (**1**, Fig. 1) and DA (**2a**), and the respective 3,4-*O*-diacetyl ester derivative (**2b**), were covalently linked through a carbamoyl group (**4a**,**b**; Scheme 1). In addition, one LD carbamate ester (**4c**) and two amide derivatives (**6a**,**b**; Scheme 2) of the OXA hemisuccinate **5** were synthesized and tested for their hydrolytic stability. These molecules were synthesized taking advantage of two features concerning benzodiazepine compounds. The first one relates to the lipophilic nature of the benzodiazepine moiety that could serve as carrier for DA (or LD derivatives) leading to increased brain levels. In this case, the benzodiazepine itself is the lipophilic pro-moiety of the hydrophilic neurotransmitter DA or LD ethyl ester. The second one is the high affinity for the GABA-benzodiazepine receptor complex shown by OXA **1** and its hemisuccinate **5**. Synthesis, hydrolytic stability, assessment of permeability-related properties, including *in vitro* transport assays for estimating BBB penetration, and *in vivo* rat brain microdialysis are herein reported. The

striatum, which is rich in dopaminergic nerve terminals, is the main target region for symptomatic treatment of PD. Therefore, we have evaluated the pharmacological effects of oxazepam-DA conjugates in the striatum of intact rats.

## 2. Materials and methods

### *2.1 Synthesis*

Melting points were determined in open capillary tubes with a Buchi apparatus and are uncorrected. Final compound purities were assessed by elemental analyses (C, H, N), performed on Euro EA3000 analyzer (Eurovector, Milan, Italy) by the Analytical Laboratory Service of the Department of Pharmacy−Drug Sciences of the University of Bari (Italy), and the results agreed to within ± 0.40% of theoretical values. IR spectra were recorded on a Perkin-Elmer Spectrum One Fourier transform infrared spectrophotometer (Perkin-Elmer Ltd., Buckinghamshire, U.K.), and the most significant absorption bands are listed. $^{1}$H NMR spectra were recorded at 300 MHz on a Varian Mercury 300 instrument. Chemical shifts are expressed in $\delta$. The following abbreviations are used: s, singlet; d, doublet; t, triplet; m, multiplet; br, broad signal. Signals due to NH and OH protons were located by deuterium exchange with $D_2O$.[20] Mass spectra were obtained by Agilent 1100 Series LC-MSD Trap System VL, equipped with ESI (electrospray ionization) source (Agilent Technologies Italia S.p.A., Cernusco sul Naviglio, Milan, Italy). Chromatographic separations were performed on silica gel 60 for column chromatography (Merck 70−230 mesh). All the reactions were carried out under a nitrogen atmosphere and the progress of the reaction was monitored by thin-layer chromatography (TLC) by using Kieselgel 60 F254 (Merck) plates. Unless otherwise

stated, starting materials and all chemicals and solvents were purchased from Sigma-Aldrich (Milan, Italy).

Dopamine 3,4-*O*-diacetyl ester derivatives (**2b**), L-Dopa ethyl ester hydrochloride (**2c**) and (±) oxazepam hemisuccinate (**5**) were prepared according to known procedures[21,22]; their melting points and spectral data were in full agreement with those reported in literature. No effort was made at this stage to optimize the synthesis yields.

*2.1.1. Synthesis of compounds **4a-c***

To a stirred solution of *rac*-oxazepam **1** (0.35 mmol) in anhydrous THF (10 ml), TEA (0.42 mmol) and bis-(4-nitrophenyl) carbonate (0.7 mmol) were added. The reaction mixture was stirred at room temperature under $N_2$ atmosphere for 3 h. The progress of the reaction was monitored by thin layer chromatography (TLC) using petroleum ether/ethyl acetate 1:1 (v/v) as the eluent. Subsequently, a solution of **2a-c** in dry DMF was added, and stirring was prolonged for 12-24 h. The progress of the reaction was monitored by TLC using petroleum ether/ethyl acetate 2:8 (v/v) as the eluent. The solvent was then removed under reduced pressure and the residue was purified by flash silica gel column chromatography using petroleum ether/ethyl acetate 1:1 (v/v) as the eluent to yield the desired conjugates **4a-c**.

**4a**: 45% yield; mp 83-85 °C; IR (KBr): 3367, 1699 $cm^{-1}$; $^1H$ NMR (DMSO-$d_6$) $\delta$: 2.5 (m, 2 H, $CH_2Ar$), 3.1 (m, 2 H, $NHCH_2$), 5.67 (s, 1 H, CH), 6.4-6.7 (m, 3 H, Ar), 7.2-7.35 (m, 2 H, Ar), 7.4-7.55 (m, 4 H, Ar), 7.69 (m, 1 H, Ar), 7.8 (br, 1 H, NH), 7.93 (s, 1 H, Ar); MS $ESI^+$ m/z: 488.0 $[M+Na]^+$; MS $ESI^-$ m/z: 463.8 $[M-H]^-$.

**4b**: 10% yield; mp 133-134 °C; IR (KBr): 1770, 1720 $cm^{-1}$; $^1H$ NMR ($CDCl_3$) $\delta$: 2.2 (s, 6 H, $CH_3$), 2.85 (t, 2 H, $CH_2Ar$), 3.46 (m, 2 H, $CH_2$), 5.95 (s, 1 H, CH), 6.4-6.7 (m, 3 H, Ar), 7.4-7.8 (m, 8 H, Ar), 9.3 (br, 1 H, NH); MS $ESI^+$ m/z: 571.9 $[M+Na]^+$; MS $ESI^-$ m/z: 547.7 $[M-H]^-$.

**4c**: 14% yield; 146-149 °C; IR (KBr): 3300, 1700 $cm^{-1}$; $^1H$ NMR ($CDCl_3$) $\delta$: 1.05 (t, 3 H, $CH_3$), 3.2 (m, 2 H, $CH_2$), 4.1 (m, 2 H, $CH_2$), 4.2 (m, 1 H, CH), 6.0 (s, 1 H, COCHN), 6.6-7.2 (m, 3 H, Ar), 7.18-7.75 (m, 6 H, CHAr), 8.63 (m, 2 H, CH Ar), 9.8 (br, 1 H, NH). MS $ESI^+$ m/z: 560.0 $[M+Na]^+$; MS $ESI^-$ m/z: 535.8 $[M-H]^-$.

*2.1.2. Synthesis of compound **6a***

A solution of oxazepam hemisuccinate **5** (0.26 mmol) and CDI (0.39 mmol) in anhydrous DMF (10 ml) was stirred at room temperature under $N_2$ atmosphere for 15 min. Then **2a** (0.39 mmol) was added, and stirring was prolonged for 12 h. The reaction was monitored by TLC using petroleum ether/ethyl acetate 2:8 (v/v) as the eluent. The solvent was removed under reduced pressure and the residue purified by flash silica gel column chromatography using petroleum ether/ethyl acetate 2:8 (v/v) as the eluent to yield **6a**.

**6a**: 12% yield; mp 99-102 °C; IR (KBr): 3400 $cm^{-1}$, 1700 $cm^{-1}$;$^1H$ NMR (DMSO $d_6$) $\delta$: 2.9 (m, 4 H, $OCOCH_2$), 3.1 (m, 2 H, $CH_2$), 4.1 (m, 2 H, $CH_2OCO$), 5.95 (s, 1 H, N-CH-CO), 6.6-7.2 (m, 3 H, CH Ar.), 7.1-7.75 (m, 8 H, CH Ar.); MS $ESI^+$ m/z: 544.0 $[M+Na]^+$; MS $ESI^-$ m/z: 520.0 $[M-H]^-$.

*2.1.3. Synthesis of compound **6b***

EDC (0.62 mmol) was added to a stirred and cooled (on ice bath) solution of **5** (0.52 mmol) in 20 ml of $CH_2Cl_2$. After 30 min, a solution of **2b** in 5 ml of $CH_2Cl_2$ and then TEA (0.78 mmol) were added dropwise. Stirring was continued for 4 h at room temperature and the progress of the reaction was monitored by TLC using petroleum ether/ethyl acetate 2:8 (v/v) as the eluent. The solvent was then removed under reduced pressure to yield compound **6b**.

**6b**: 32% yield; mp 120-123 °C; IR (KBr): 3380 $cm^{-1}$, 1760 $cm^{-1}$, 1740 $cm^{-1}$, 1700 $cm^{-1}$, 1650 $cm^{-1}$; $^1H$ NMR ($CDCl_3$) δ: 2.0 (s, 6 H, OCO-$CH_3$), 2.5-3.0 (m, 6 H, $CH_2$), 5.95 (s, 1H, CH), 6.6 (t, 1 H, NH), 7.0-7.6 (m, 11 H, CH Ar); MS $ESI^-$ m/z: 603.8 $[M-H]^-$.

*2.2. Stability studies*

*2.2.1. Chemical hydrolysis*

The chemical hydrolysis of each test compound was monitored at pH 7.4 in 0.05 M phosphate buffer (PBS) at 37 ± 0.5 °C using a shaking water bath. Reactions were initiated by adding 50 µl of a DMSO stock solution to 5 ml of the preheated buffer solution. The resulting final concentration was 50 µM. The test solutions were vortexed and kept in a shaking water bath at constant temperature (37 ± 0.5 °C). Pseudo-first-order rate constants ($k_{obs}$) for the hydrolysis of the compounds were then calculated from the slopes of the linear plots of log (% residual compound) against time.[23,24] The % residual compound was measured by HPLC at several time intervals. The experiments were run in triplicate.

*2.2.2. Serum hydrolase-catalyzed hydrolysis*

Fetal bovine serum (FBS) was diluted with 0.05 M PBS (pH 7.4, 0.14 M NaCl) to 50% v/v, and incubation of each test compound was performed at 37 ± 0.5 °C using a shaking water bath. The reaction was initiated by adding 10 µl of a stock solution of the test compound in DMSO (10 mM) to 1.6 ml of the preheated FBS solution. Aliquots (100 µl) were withdrawn at various times and deproteinized by mixing with 500 µl of cold acetonitrile (MeCN). After centrifugation for 10 min at 3500 rcf, 10 µl of the supernatant was analyzed by HPLC. Pseudo-first-order rate constants for the disappearance of the test compounds were determined from the slope of the linear plots of the log (% residual compound) against time. Degradation products of the test compounds in FBS were analyzed by direct injection of an aliquot of deproteinized supernatant (further diluted 1:10 with blank) into an ESI mass spectrometer (both positive and negative mode).

The stability of **4a** and **4c** was also assessed in 100% rat and human serum (lyophilized and reconstituted with 4 ml of deionized water). The reaction was initiated by adding 10 µl of a DMSO stock solution of the test compound (10 mM) to 0.990 ml of preheated serum, and incubation was performed at 37 ± 0.5 °C using a shaking water bath. At appropriate time intervals, aliquots (200 µl) were withdrawn and deproteinized by mixing with 0.800 ml of ice-cold MeCN. After centrifugation for 5 min at 3500 rcf, the supernatant was filtered (0.2 µm PTFE membrane) and analyzed by HPLC. The percentage of the residual compound was measured by monitoring the peak area of the chromatogram (external standard).

*2.2.3. HPLC analyses*

The HPLC analyses for monitoring the disappearance of the starting test compounds were carried out at temperature of 25 ± 0.2 °C and UV detection at 254 nm wavelength, using an Agilent 1260 infinity HPLC system equipped with a diode array detector (Agilent Technologies Italia). The analyses were performed on a Phenomenex Kinetex C18 column (150 × 4.6 mm i.d.; 5 µm particles), using MeOH and 10 mM ammonium formate solution (pH 5.0) mixed in different fraction composition (typically, 60:40 v/v, respectively) depending upon the retention of the analyte (injection volume: 10 µl; flow rate: 1 ml/min). Calibration curves were obtained by measuring peak areas for each test compound at known concentrations.

The stability in rat serum of the enantiomers of racemic **4a** has been monitored using as the chiral stationary phase a Chirobiotic TAG steel column (250×4.6 mm i.d., 5 µm particles), equipped with the teicoplanin aglycone bonded to silica gel (Astec, Whippany, NY, USA). The **4a** enantiomers were eluted in polar mode using MeOH as the mobile phase (injection volume: 2 µl; flow rate 0.3 ml/min).

*2.3. Lipophilicity and blood-brain barrier permeability descriptors*

Lipophilicity was assessed by calculated log P (octanol/water partition coefficient) using ClogP software (v. 4, BioByte Corp.). Polar surface area, that is the surface area in $Å^2$ occupied by nitrogen and oxygen atoms, and the connected polar hydrogens, was estimated using a simple protocol proposed by Ertl et al. for calculating the so-called topological PSA (tPSA).[25] The likelihood that a compound penetrates the blood-brain barrier (BBB) was estimated by calculating $\log(C_{brain}/C_{blood})$, expressed as logBB value, according to Clark's equation[26]:

$$\text{LogBB} = -0.0148\ (\pm 0.001)\ \text{PSA} + 0.152\ (\pm 0.036)\ \text{ClogP} + 0.139\ (\pm 0.073) \quad (1)$$

*2.4. Cell cultures and transport studies on MDCKII-MDR1 monolayers*

Cell cultures were grown under controlled conditions as previously described by Denora et al.[21] The blood-brain transport of compounds was investigated using MDCKII-MDR1 cell monolayers (passage 8-11). Cells were seeded at a density of 100,000 cell/cm$^2$ onto polyester Transwell inserts (pore size 0.4 µm, diameter 6.5 mm, apical volume 0.5 ml, basolateral volume 1.5 ml). MDCKII-MDR1 cell barrier function was verified prior to the transport experiments by means of trans-epithelial electrical resistance (TEER) using an EVOM apparatus and the measurement of the flux of FD4 and diazepam as paracellular and transcellular markers of cell monolayers integrity. The TEER was measured in growth media (DMEM) at room temperature and calculated as the measured resistance minus the resistance of an empty Transwell (blank without cells). Cell monolayers with TEER values above 200 Ohm cm$^2$ were used. Following the TEER measurements, the cells were equilibrated in transport medium in both the apical and basolateral chambers for 30 minutes at 37 °C. At time 0, a solution of compounds diluted in the transport medium was added to the apical chamber. The transport experiments were carried out under cell culture conditions (37 °C, 5% $CO_2$, 95% humidity) with constant stirring (70 rpm). At 15 min, 30 min, 45 min, 1 h, 1 h and 30 min, 2 h, 3 h, 200 µl samples were removed from the basolateral chamber and replaced with 200 µl of fresh transport medium. At the final time point, a 200 µl sample was also removed from the apical chamber. The transport of compounds was investigated at a concentration of 50

μM. The apparent permeability coefficient ($P_{app}$) was determined according to the following equation:

$$P_{app} = \frac{V \cdot dC}{A \cdot C_0 \cdot dt} \quad (2)$$

where A is the filter/cell surface area, $C_0$ is the initial concentration of the test compound **4a**, and V(dC/dt) is the linear appearance rate of mass in the receiver solution. Concentrations of **4a**, FD4, diazepam and dopamine were assessed by HPLC.

The experiments were performed in triplicate and results are expressed as means ± SEM. Statistical analysis was carried out using Student's t-test between two mean values.

*2.5. Brain microdialysis*

*2.5.1. Animals*

Animal care and all experiments were conducted in accordance with the guidelines of the European Communities Council Directive of 24 November 1986 (86/609/EEC) and the Italian Department of Health (D.L. 116/92), and approved by the Institutional Animal Care and Use Committees of the University of Foggia, Italy. All efforts were made to minimize animal suffering and to reduce the number of animals used in the study. Male Wistar rats (225-250 g; Harlan, San Pietro al Natisone, Udine, Italy) were housed on 12-h dark-light cycle, at 22 ± 1° C with food and water available *ad libitum* and habituated to housing conditions for 1 week before the experiments.

*2.5.2. Microdialysis and treatment schedule*

*In vivo* microdialysis experiments were carried out as previously described.[18,27] Briefly, animals were anesthetized with equithesin (3 ml/kg, i.p.) and a custom-made

microdialysis probe of concentric design (AN69 Hospal S.p.A; 20 kDa cut-off; membrane length, 3-mm) was implanted stereotaxically into the striatum according to the following stereotaxic coordinates: AP +1.0, ML ±2.8 from bregma and DV -6.8 from dura.[28] The membranes were tested for in vitro recovery of DA on the day before surgery to verify that recoveries were within a desired range (~ 30 % of DA). Twenty-four hours after surgery, the microdialysis probe was perfused with Krebs Ringer solution (NaCl 145 mmol/l, KCl 2.7 mmol/l, $CaCl_2$ $2H_2O$ 1.2 mmol/l, $MgCl_2$ $6H_2O$ 1 mmol/l, $Na_2HPO_4$ 2 mmol/l, pH 7.4) at a constant flow rate of 2 µl/min. Perfusates were collected every 20 min into mini-vials. After a wash-out period of 2 hours, 4 samples were collected to determine the baseline levels of the DA (no more than 10% difference among 4 consecutive samples).

Compound **4a** was freshly dissolved in saline/Tween 80/PEG (90/5/5) and administered i.p. in a volume of 3 ml/kg. Rats were acutely treated with **4a** (10 and 20 mg/kg, i.p.) or vehicle and consecutive microdialysate samples were collected every 20 min over a 4 h period.

A second set of animals was treated with a single injection of LD + the inhibitor of aromatic aminoacid decarboxylase, benserazide (8.5 mg/kg and 12.5 mg/kg i.p., respectively) or vehicle (saline), and dialysates were collected over a 2-h period. DA concentration, obtained from the same samples, were detected and quantified by HPLC. At the end of each experiment, the correct placement of dialysis probes was verified histologically.

*2.5.3. HPLC Analyses*

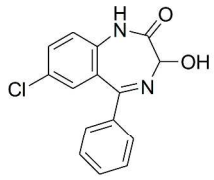
The extracellular level of DA was determined by HPLC using a ODS Hypersil column (150×3 mm i.d.; 5 μm particles) (Thermo Fisher Scientific, Milan, Italy) with an Unijet cell (BAS, Bioanalytical Systems, Kenilworth Warwickshire, United Kingdom) equipped with a 6-mm-diameter glassy carbon electrode (set at + 650 mV) and connected to an electrochemical amperometric detector (INTRO, Antec Leyden, The Netherlands), as previously described.[27,29] The flow rate of the mobile phase (85 mM sodium acetate, 0.34 mM EDTA, 15 mM sodium chloride, 0.81 mM octanesulphonic acid sodium salt, 5% methanol (v/v), pH 4.85) was 0.7 ml/min and the total runtime 15 min.

*2.5.4. Statistical analysis*

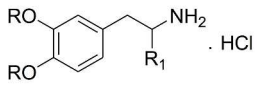
Neurochemical data were expressed as percentages of baseline (± S.E.M.), which was defined as the average of four consecutive samples with stable level of neurotransmitters before drug administration. Data were analyzed by two-way analysis of variance (ANOVA) for repeated measures with treatment (tr) as the between variable and time (t) as the within variable. Violations of the sphericity assumption were corrected using the Greenhouse-Geisser epsilon correction to adjust the degrees of freedom for each test. Post-hoc test was made by Bonferroni's multiple comparison test. The overall effect of drug treatments on the striatal DA outputs was estimated by one-way ANOVA and by comparing the areas under the curve (AUC) by the un-paired Student's t-test. The AUC was calculated using the standard trapezoid method[30] using neurotransmitter levels over a time window of 0–240 min for each experimental group. The threshold for statistical significance was set at $P < 0.05$.

## 3. Results and discussion

Based on previous findings[18,21], in this study the neurotransmitter DA and its 3,4-*O*-diacetyl ester derivative were coupled with OXA **1** (Fig. 1) through carbamate linkage (**4a** and **4b**) and succinic spacer (**6a** and **6b**).



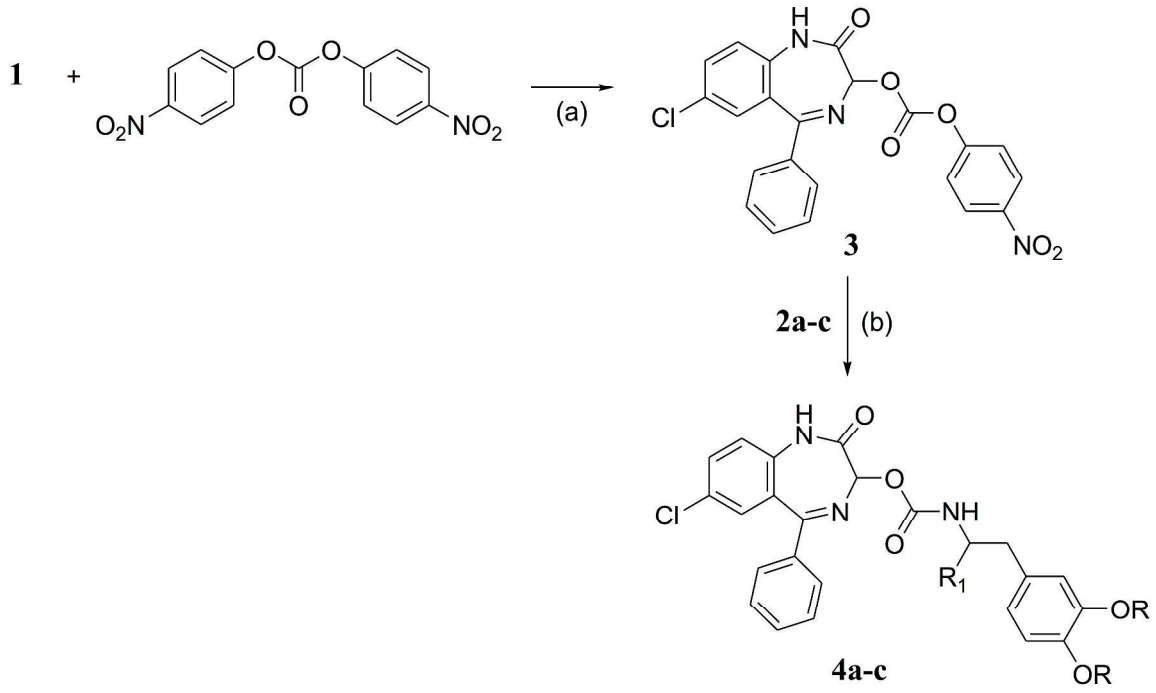
**1**, oxazepam



**2a**, R = $R_1$ = H (dopamine)
**2b**, R = $COCH_3$, $R_1$ = H
**2c**, R = H, $R_1$ = $COOCH_2CH_3$

**Fig. 1.** Structures of *rac*-oxazepam (**1**), HCl salts of dopamine (**2a**) and its 3,4-*O*-diacetyl ester (**2b**), and L-Dopa ethyl ester (**2c**).

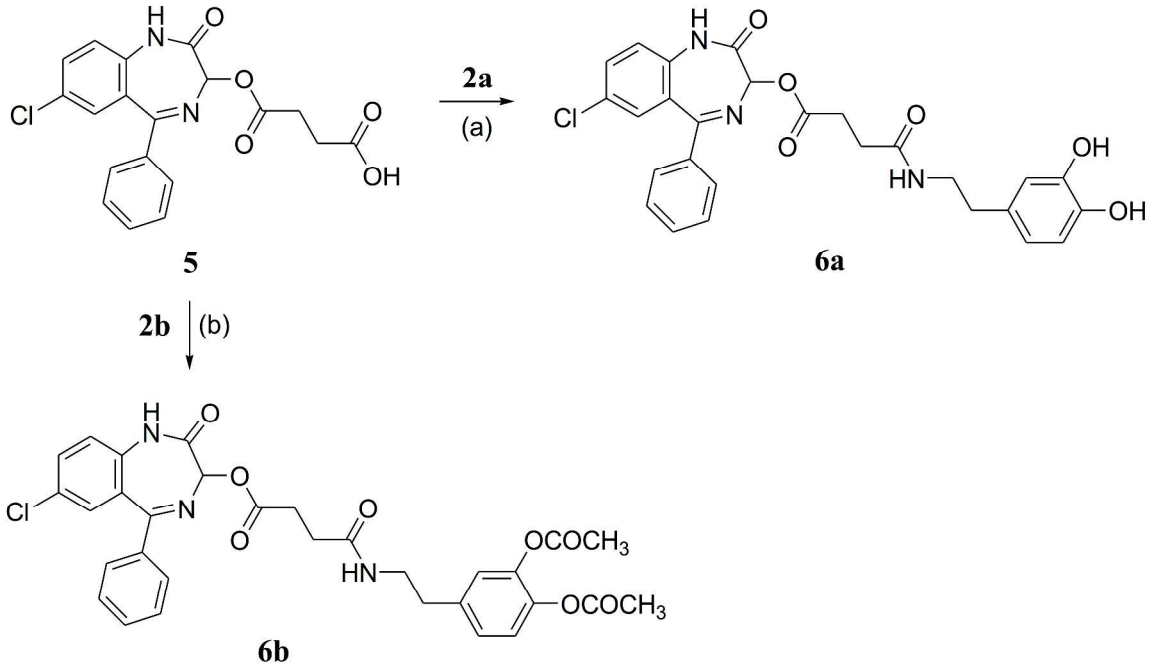
As outlined in Scheme 1, compounds **4a** and **4b** were synthesized through the formation of the carbonate intermediate **3** and subsequent reaction with **2a**,**b** in dry DMF.



**Scheme 1**. Synthesis of oxazepam-dopamine (L-DOPA) carbamoyl derivatives. Reagents and conditions: (a) Dry THF, TEA, $N_2$ atm., r.t., 3 h; (b) dry DMF, r.t., 12-24 h.

The starting 3,4-*O*-diacetyl ester of DA (**2b**) was prepared in almost quantitative yield by treating DA, dissolved in anhydrous trifluoroacetic acid, with acetyl chloride. This method, that is a modification of the classic acetylation of catechol OH groups, provided satisfactory yields, due to the dual advantage of the simplicity of direct acetylation of OHs (i.e., without the preparation of *N*-carbobenzyloxy derivative of DA) and higher yield. The conjugate **4c** was similarly prepared by reacting LD ethyl ester **2c** with **3**. Unfortunately, because of their low stability, chromatographic purification of the end products **4b** and **4c** on a normal phase silica column decreased the total yields compared to compound **4a**.

The DA-amide derivatives **6a** and **6b** of OXA hemisuccinate **5** were synthesized using CDI and EDC as suitable coupling agents, respectively (Scheme 2).



**Scheme 2**. Synthesis of oxazepam hemisuccinate-dopamine amide derivatives. Reagents and conditions: (a) CDI, dry DMF, $N_2$ atm., r.t., 15 min; then, **2a**, r.t., 12 h; (b) EDC, $CH_2Cl_2$, 0 °C, 30 min; then, **2b**, $CH_2Cl_2$, TEA, r.t., 4 h.

The BBB penetration of the five synthesized conjugates was estimated using the Clark's model[26], a structure-based quantitative property relationship relating logBB (i.e., log blood-brain partitioning) to calculated log P and PSA (Table 1). As a general trend, compounds with logBB < -1.0 may be unable to enter the CNS, whereas compounds with values 0.3-0.5 should cross to some extent the BBB and compounds with values > 1.0 should have a desirable BBB permeability. The logBB values of the newly synthesized conjugates were all lower than -1, suggesting a low probability for crossing the BBB. However, this can not lead to rule out that at least **4a**, the conjugate showing the highest predicted logBB, may penetrate the BBB.

An ideal brain-targeted prodrug or codrug should have a sufficient linker stability in plasma to enable the conjugate to circulate intact in the bloodstream before penetrating and distributing within the CNS. Once the intact conjugate molecule permeated the BBB, the linker should be labile enough within the brain to efficiently release the active compound/s, such as DA and OXA in this case.

**Table 1**

Lipophilicity, blood-brain permeability and stability of the OXA-DA conjugates.

| Cmpd | ClogP[a] | tPSA[b] | Log BB[c] | Half-life (37°C)[d] | |
|---|---|---|---|---|---|
| | | | | PBS (pH 7.4), h | FBS, min |
| **4a** | 3.51 | 120.25 | -1.11 | 24.3 ± 0.2 | 145 ± 23 |

| | | | | | |
|---|---|---|---|---|---|
| **4b** | 3.30 | 132.39 | -1.32 | 7.52 ± 0.50 | 15.4 ± 1.2 |
| **4c** | 3.67 | 146.55 | -1.47 | 9.13 ± 0.30 | 26.9 ± 0.1 |
| **6a** | 3.01 | 137.32 | -1.44 | 1.70 ± 0.14 | 68.2 ± 16.2 |
| **6b** | 2.79 | 149.46 | -1.65 | 1.19 ± 0.01 | 9.40 ± 0.10 |

[a] Calculated logP using ChemDraw Ultra 10.0 software.

[b] Topological polar surface area calculated according to Ertl's equation.[25]

[c] Log ($C_{brain}/C_{blood}$) calculated according to Clark's equation.[26]

[d] Stability evaluated in 0.05 M phosphate buffer solution (PBS, pH 7.4, 0.15 M KCl) and diluted fetal bovine serum (FBS) solution (50%, v/v) at 37 °C; half-life, $t_{1/2}$ (mean ± SEM, $n$ = 3), refers to disappearance of the starting compound and is expressed in hours (h) and minutes (min), respectively.

The study of chemical and enzymatic stability was performed at 37 °C in 0.05 M PBS and in FBS solution (the most widely used serum-supplement for the in vitro cell culture of eukaryotic cells), respectively, at pH 7.4. In both media, all the test compounds disappeared following pseudo-first-order hydrolysis kinetics, and the observed half-lives ($t_{½}$) are reported in Table 1. Although the degradation products of the carbamate-containing compounds **4a-c** and hemisuccinate amide derivatives **6a-b** were not fully characterized, HPLC analyses, carried out at regular intervals along the monitoring time between $t_0$ and $2\times t_{½}$, were combined with ESI MS. In the chromatographic conditions used, the HPLC chromatograms of the test compounds incubated both in PBS and diluted FBS at pH 7.4 revealed several degradation products. In particular, the main fractions collected by HPLC and analyzed by ESI MS in negative mode revealed the appearance of OXA **1** (m/z 285) as a major hydrolytic decomposition product from **4a** and **4c**, compound **4a** (the deacetylation product) and **1** from **4b**. OXA **1**, and to a lesser extent **5** (m/z 385), were revealed as the hydrolysis products of **6a** and **6b**. The retention times of

the observed degradation products were also confirmed in HPLC by comparison to the standards, suggesting that the hydrolysis at the carbamate bond in compounds **4** as well as at the ester and amide bonds in compounds **6** occurred with different rates in PBS and FBS at physiological pH and temperature.

The study of the stereoselective (enantio- and diastereoselective) enzyme-catalyzed hydrolysis is out of the purposes of this work, given that the examined conjugates could release by hydrolysis as major active products only the achiral DA (**2a**), or L-DOPA from **4c**, and OXA **1** in racemic form (*rac*-OXA). On the other hand, it is known that the *R* and *S* enantiomers of OXA can undergo racemization (bidirectional chiral inversion at C3) in aqueous solution *in vitro*.[31,32] The half-lives in Table 1 refer to disappearance of racemic **4a**, **4b**, **6a** and **6b**, and diastereomeric mixture of **4c** (i.e., sum of the peak areas of the two diastereoisomers).

Compounds bearing the succinic spacer (**6a** and **6b**) proved to be in PBS much less stable than the respective carbamate derivatives (**4a** and **4b**), and the hydrolytic disappearance of the 3,4-*O*-diacetyl ester derivatives **4b** and **6b** occurred faster than the parent compounds **4a** and **6a**. The disappearance in PBS of the LD ethyl ester conjugate **4c** ($t_{1/2}$ = 9.13 h), due to hydrolysis of the $COOCH_2CH_3$ group, was about three-fold faster than the respective DA conjugate **4a** ($t_{1/2}$ = 24.32 h).

The enzyme-catalyzed hydrolysis of the carbamate-based conjugates **4** in FBS proved to be ten-to-thirty faster than in pH 7.4 aqueous solution and comparatively much less effective for the OXA hemisuccinate derivatives **6**. Compound **4a**, with half-life slightly more than two hours, proved the most stable one in FBS. The stability of **4a**, and **4c** for comparison, was also studied in rat and human sera at 37 °C (Table 2).

**Table 2**

Average half-lives of **4a** and **4c** in 100% rat and human serum at 37 °C.

| | Rat (min) | Human (h) |
|---|---|---|
| **4a** | 109 | 6.10 |
| **4c** | < 1 | 11.3 (12.4) [a] |

[a] Data in parentheses refer to the second (more retained) diastereoisomer of **4c**.

The rate of hydrolysis of **4a** to produce DA and *rac*-OXA was just 1.3-fold faster in 100% rat serum than in diluted FBS. In contrast, its half-life in 100% human serum exceeded 6 h. This so marked difference in serum stabilities could depend upon the different ester hydrolase activities in human compared to rodent serum.[33,34] Indeed, rats and humans differs in serum esterase composition, mainly for carboxylesterases which are present in rat serum and not in human serum. That carboxylesterases can effectively contribute to catalyze the hydrolysis of the compounds under examination is also shown by the decomposition rate of the double ester **4c**, which is hydrolyzed in rat serum with a $t_{1/2}$ of less than 1 min and highly stable in human serum ($t_{1/2}$ > 11 h).

A preliminary enantioselective HPLC determination of *rac*-**4a** at several times in rat serum showed that the two enantiomers should be hydrolyzed with very similar rates, their half-lives resulting 135 and 126 min (Fig. 2).

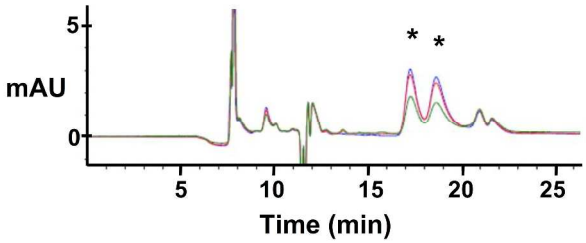


**Fig. 2.** Overlay of chromatograms for the chiral separation of *rac*-**4a** in rat serum at different incubation times: $t$ = 0 min (blue), $t$ = 60 min (magenta), $t$ = 120 min (green). The chiral stationary phase resolved the enantiomers (*) with peaks at 17.50 and 18.90 min. Column: Astec CHIROBIOTIC TAG, 250×4.6 mm i.d., 5 µm particles (Whippany, NY, USA). Mobile phase: Methanol. Column temperature: 25 °C. Flow rate: 0.3 ml/min. Detection: UV, 254 nm. Injection: 2 µl.

Stability data in Tables 1 and 2 suggest that the carbamate-based conjugate **4a** could be stable enough in the circulatory system to cross intact the BBB (the average half-live of **4a** in 100% human serum at 37 °C is 6.1 h), possibly releasing DA and OXA in the brain parenchyma after hydrolysis. Compound **4a**, showing the highest plasma stability and logBB value was screened for its permeability across a BBB model.

The ability of **4a** to cross the BBB was furnished by *in vitro* transport assays performed with MDCKII-MDR1 monolayers, a cell model that has been found to be predictive of passive BBB permeability. Data in Table 3 supported the ability of **4a** to cross the BBB; **4a** has a $P_{app}$ value 20-fold higher than that of DA (**2a**), only 3-fold lower than $P_{app}$ of diazepam, and about 7-fold greater than that of FD4 (a marker for the paracellular pathway).

**Table 3**

MDCKII-MDR1 permeability value of compound **4a** compared to those of dopamine (**2a**), diazepam and FD4.

| Compound | $P_{app}\times10^{-6}$(cm/sec) [a] |
|---|---|
| **4a** | 6.73 ± 0.82 |
| **2a** | 0.39 ± 0.28 |

| | |
|---|---|
| Diazepam | 18.9 ± 1.2 |
| FD4 | 0.977 ± 0.292 |

[a] Mean ± SEM, $n = 3$.

To assess whether the peripheral (i.p.) administration of **4a** affects brain dopaminergic transmission, *in vivo* microdialysis experiments were performed to determine the extracellular output of DA in the striatum of freely-moving rats. To this regard, since long time *in vivo* microdialysis has been increasingly applied to monitor the penetration of various agents across the BBB.[35] Continuous perfusion of the probe allowed the sequential collection of samples of dialysate containing, among other solutes in the extracellular fluid, the neurotransmitters released by neurons so that changes in their concentrations provides information on the effects of centrally acting drug treatment.[35] According to our experimental protocol, a series of dialysis samples was first obtained for determination of basal neurotransmitter levels and then vehicle and drug solutions (**4a** or LD) were administered. In particular, the effects of single injections (10 and 20 mg/kg, i.p.) of **4a** on the basal extracellular concentrations of DA (524 ± 80 pg/ml, value not corrected for *in vitro* probe recovery) were monitored for 4 h after administration (Fig. 3 A). Two-way ANOVA statistics revealed a significant main effect of treatment ($F_{(2,154)} = 4.782$, $P < 0.05$), a significant main effect of time ($F_{(14,154)} = 3.232$, $P < 0.001$), and any significant interactions between factors ($F_{(28,154)} = 1.499$, n.s.). The post-hoc test showed that **4a** evoked a dose-dependent increase of DA extracellular outflow in the striatum. Following the administration of the highest dose of **4a** (20 mg/kg, i.p.), a significant increase of DA level was observed at 60 min after administration that peaked (1663 % increase above the baseline) at 80 min and disappeared at 120 min after treatment. On the

contrary, neither lower dose of **4a** (10 mg/kg, i.p.) or vehicle treatment had any significant effect on striatal DA release (Fig. 3 A, B).

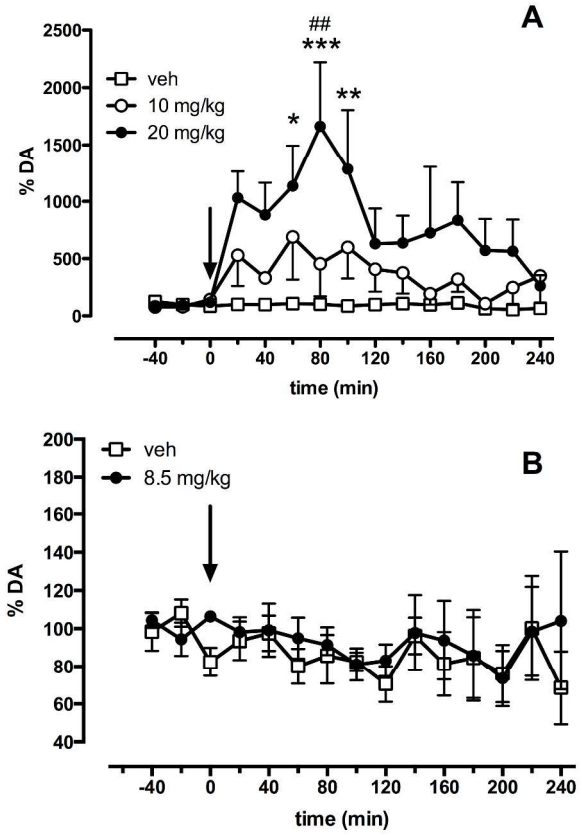



**Fig. 3.** A) Effect of single injections of **4a** (10 and 20 mg/kg, i.p.) on the DA outflow in the striatum of awake rats over 4 h. Data are expressed as mean percentages of baseline (± SEM), n = 4-5 animals per group; two-way ANOVA followed by Bonferroni's multiple comparison test: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ *vs.* respective vehicle; $^{\#\#}$ $P < 0.01$ *vs.* respective **4a** lower dose (10 mg/kg). Arrow indicates the time of **4a** or vehicle administration. B) Effect of single injections of LD (8.5 mg/kg, i.p.) and vehicle (saline) on the DA outflow in the striatum of awake rats over 4 h. Data are expressed as mean percentages of baseline (± SEM), n = 5 animals per group. Statistical analysis was performed by two-way ANOVA. Arrow indicates the time of LD or vehicle administration.

The brain microdialysis study has been carried out also with the golden standard treatment of PD. A second set of animals was treated with LD (8.5 mg/kg, i.p.), co-administered with the inhibitor of aromatic aminoacid decarboxylase benserazide (12.5 mg/kg, i.p.), and the extracellular level of DA in the striatum was monitored. The dose of LD was equimolar to the higher dose of **4a** (20 mg/kg) that significantly increases the level of DA. Statistical analysis showed that LD did not modify the extracellular level of DA in the striatum compared to vehicle-treated rats ($F_{time(14,112)}$ = 0.8605, n.s., $F_{treatment(1,112)}$ = 0.2847, n.s., $F_{time\ x\ treatment(14,112)}$ = 0.4143, n.s.) (Fig. 3 B, n=5).

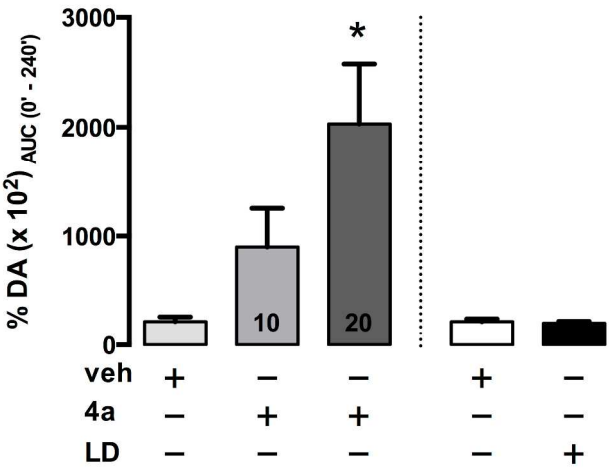



**Fig. 4.** AUC values of striatal dopamine outflow after single injection of vehicle, **4a** (10 and 20 mg/kg. i.p.) or LD (8.5 mg/kg, i.p.) in freely moving rats. Statistical analysis was performed by one-way ANOVA followed by Bonferroni's multiple comparison test: * $P < 0.05$ *vs.* respective vehicle.

The overall effect of treatments was assessed by comparing their respective AUCs (Fig. 4). Statistical analysis confirmed that only **4a** at the highest dose significantly increases

the striatal levels of DA (+ 90% compared to vehicle-treated rats). The lowest dose of **4a** increases the DA output in the striatum up to 75%, although it did not attain statistical significance. Conversely, the dose of LD, equimolar to the highest dose of **4a,** did not increase DA release in the striatum. The latter results are in line with our previous microdialysis experiments, where we found no increase in striatal DA efflux immediately after a single LD/benserazide injection (6 mg/kg and 12.5 mg/kg, s.c., respectively) in rats treated acutely or in rats that had been treated chronically with LD/benserazide (6 mg/kg and 12.5 mg/kg, s.c., respectively) for 11 days.[27]

In the literature, a wide range of LD dosages has been reported in microdialysis experiments (from 6 up to 120 mg/kg), and an increase of the extracellular DA levels in the striatum has been observed only with very high concentrations of LD (99 and 120 mg/kg, i.p.) compared to the concentration of **4a** used in the present study.[36]

Moreover, other authors have demonstrated that chronic treatment (21 days) with LD/benserazide (6 or 24 mg/kg and 15 mg/kg p.o., respectively) is able to produce a dose-related increase of the extracellular levels of DA only in the 6-hydroxyDA- (6-OHDA)-lesioned hemisphere of the hemi-parkinsonian rats.[37,38] These findings are in line with the results of many other groups.[36,39-41]

Keeping in mind that these doses are typically associated with a marked increment of brain LD levels[39,42-44], the low response of DA on the intact brain (or intact hemisphere in hemiparkinsonian rats) after LD systemic injection indicates that under physiological conditions dopaminergic neurons are able to handle extracellular DA very efficiently, thus keeping physiological homeostasis. Differently, DA availability markedly increased

(+ 600%) after DAergic denervation of the striatum, indicating a disruption of the normal regulation of extrasynaptic DA.[36]

From the present investigation and several *in vivo* microdialysis studies, it is clear that the peripherally administered LD, irrespective of the administration route (i.p., s.c. or p.o.), does not increase the extracellular DA release in the striatum of intact rats, at least at doses comparable with those tested for **4a**. It will be interesting in the next future to compare the effects of the new conjugate **4a** with those of LD in models of 6-OHDA lesioned rats.

As a matter of facts, following i.p. administration of **4a**, DA levels significantly increased in striatum at the higher concentration (20 mg/kg), ultimately supporting the hypothesis that the OXA-DA carbamate derivative **4a**, sufficiently stable in rat serum, is able to cross the BBB and undergo hydrolytic decomposition in the brain parenchyma, achieving sustained DA levels within 3 h. This hypothesis is consistent with the behavior of previously reported imidazopyridine derivatives of DA.[18]

Although the overall results of **4a** seem promising, other preclinical studies should be conducted to further warrant the usefulness of this compound in PD therapy. As mentioned before, drugs acting as dopaminergic agonists may elicit different effects on DA extracellular release depending upon the animal model used. Therefore, in future experiments the efficacy of **4a** in 6-OHDA-lesioned rats after acute and sub-chronic treatment will be tested. The latter may be conducted with the lower dose (10 mg/kg) of **4a**, since the magnitude of DA release by the higher dose may cause side effects after sub-chronic treatment. Recent *in vitro* and *in vivo* studies have demonstrated that long term LD therapy can cause DA- or LD-induced neurotoxicity,[45,46] due to the reactive

oxygen species (ROS) or reactive nitrogen species (RNS) and quinones generated in the enzymatic oxidation or auto-oxidation of an exceeding amount of DA. Specifically, the primary consequences of DA- or LD-induced oxidative stress are depletion of endogenous antioxidants, mitochondrial dysfunction, neuronal damage, apoptotic or non-apoptotic cell death. To this regard, the use of exogenous antioxidants could scavenge ROS and RNS in the brain, preventing the DA-induced damage and/or maintaining optimal DA levels.[20,23,24,47-49]

Of note, Table 2 shows that half-lives of compounds **4a** and **4c** are higher in human serum than in rat serum. This must not be surprising, taking into account the different plasma esterases' composition in humans and rats.[33,34] A possible consequence of these findings could be that **4a**, which is more stable in human than in rat plasma, may penetrate the BBB more efficiently in humans, and be active at lower concentrations with less side effects. This is just a hypothesis that, however, points out the need for a critical examination of qualitative and quantitative differences among animal species, among humans, and between healthy people and people affected by pathological conditions.

## 3. Conclusions

In this study, we synthesized some novel compounds in which DA was covalently joined to OXA, a well-known positive allosteric modulator of $GABA_A$ receptor, through a carbamate linkage (**4**) or a succinic spacer (**6**). These potential antiparkinson codrugs have been designed with the aim of improving the delivery of the neurotransmitter DA into the brain, while enhancing GABAergic transmission. Based on the *in vitro* stability data in animal serum, and using as 'filters' for estimating the potential CNS penetration

computational descriptors and data from a cell model of transport through BBB (MDCKII-MDR1 monolayers), the OXA-DA carbamate derivative **4a** was identified as the conjugate achieving sufficient stability in rat serum and ability to cross BBB to some extent. With regard to serum stability, the kinetic data showed that no advantage could come from acetylation of the catechol OH groups in DA, given that the 3,4-*O*-diacetyl ester derivative **4b** (and **6b** as well) disappeared rapidly in serum due to the lability of the $OCOCH_3$ groups. Interestingly, brain microdialysis experiments in rats provided an *in vivo* proof that **4a** (20 mg/kg, i.p.) should diffuse passively across the BBB and release DA into the brain after enzymatic hydrolysis, with a peak (more than 15-fold increase over the baseline) at 80 min after administration. *In vivo* toxicity studies will be performed in the next future, but the stability and DA-related delivery data of this study highlight compound **4a** as a noteworthy codrug candidate for further pharmacological studies in animal models of PD, such as the hemiparkinsonian rats (i.e., rats with 6-OHDA-lesioned hemisphere), in comparison with the golden therapy with LD.

**Disclosure of interest**

The authors report no conflicts of interest.

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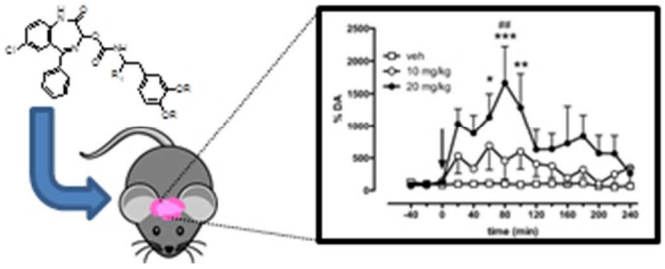


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