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A Novel Conjugated Agent between Dopamine and an A_{2A} Adenosine Receptor Antagonist as a Potential Anti-Parkinson Multitarget Approach

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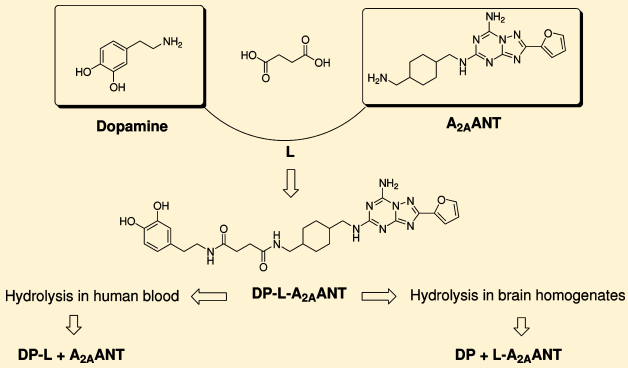
ABSTRACT: We propose a potential antiparkinsonian prodrug DP-L-A_{2A}ANT (**2**) obtained by amidic conjugation of dopamine (**1**) via a succinic spacer to a new triazolo-triazine A_{2A} adenosine receptor (AR) antagonist A_{2A}ANT (**3**). The affinity of **2** and its hydrolysis products—**1**, **3**, dopamine-linker DP-L (**4**) and A_{2A}ANT-linker L-A_{2A}ANT (**5**)—was evaluated for hA_1, hA_{2A}, hA_{2B} and hA_3 ARs and rat striatum A_{2A}ARs or D_2 receptors. The hydrolysis patterns of **2**, **4** and **5** and the stabilities of **1** and **3** were evaluated by HPLC analysis in human whole blood and rat brain homogenates. High hA_{2A} affinity was shown by compounds **2** (K_i = 7.32 ± 0.65 nM), **3** (K_i = 35 ± 3 nM) and **5** (K_i = 72 ± 5 nM), whose affinity values were similar in rat striatum. These compounds were not able to change dopamine affinity for D_2 receptors but counteracted the CGS 21680-induced reduction of dopamine affinity. DP-L (**4**) was inactive on adenosine and dopaminergic receptors. As for stability studies, compounds **4** and **5** were not degraded in incubation media. In human blood, the prodrug **2** was hydrolyzed (half-life = 2.73 ± 0.23 h) mainly on the amidic bound coupling the A_{2A}ANT (**3**), whereas in rat brain homogenates the prodrug **2** was hydrolyzed (half-life > eight hours) exclusively on the amidic bound coupling dopamine, allowing its controlled release and increasing its poor stability as characterized by half-life = 22.5 ± 1.5 min.



KEYWORDS: *A_{2A}/D_2 receptor heteromers, A_{2A} antagonist, controlled release, dopamine, HPLC, human blood, hydrolysis, prodrug, rat brain homogenates, stability*

INTRODUCTION

Parkinson's disease (PD) is a chronic neurological disorder characterized by tremors, muscular rigidity, bradykinesia, poor balance and difficulty in walking. This disease results from the progressive and irreversible degeneration of dopaminergic neurons in the substantia nigra of the brain, with a consequent depletion of dopamine production. PD is clearly revealed with a 70−80% reduction in nigrostriatal dopamine concentration.[1]

Dopamine replacement currently represents the major therapeutic strategy to alleviate the neurological symptoms associated with PD. At present, from a pharmacologiacal point of view, dopamine is administered as L-DOPA, its metabolic precursor, which is able to cross the blood−brain barrier and is converted into the active drug by enzymatic decarboxylation.[2] Even if L-DOPA is effective in ameliorating the motor symptoms of patients in the early stage of PD, important side effects seriously limit the therapeutic potential of this drug.[3] Administration of L-DOPA is associated with both acute peripheral phenomena, such as nausea, vomiting and hypotension, due to the activation of dopaminergic receptors,[4] and a series of central complications that arise during long-term use of L-DOPA, which dramatically increase in severity as the disease evolves. These complications depend on the progressive loss of the drug's ability to relieve motor impairments (wearing off), on the induction of excessive and abnormal purposeless movements, which interfere with the normal motor activity (dyskinesias), and on fluctuations in the intensity of the motor stimulation effects of L-DOPA ("on/off phenomena").[5] The mechanisms triggering these phenomena are not clearly understood, even if they are attributed to abnormal permanent plastic changes in striatal synapses induced by L-DOPA

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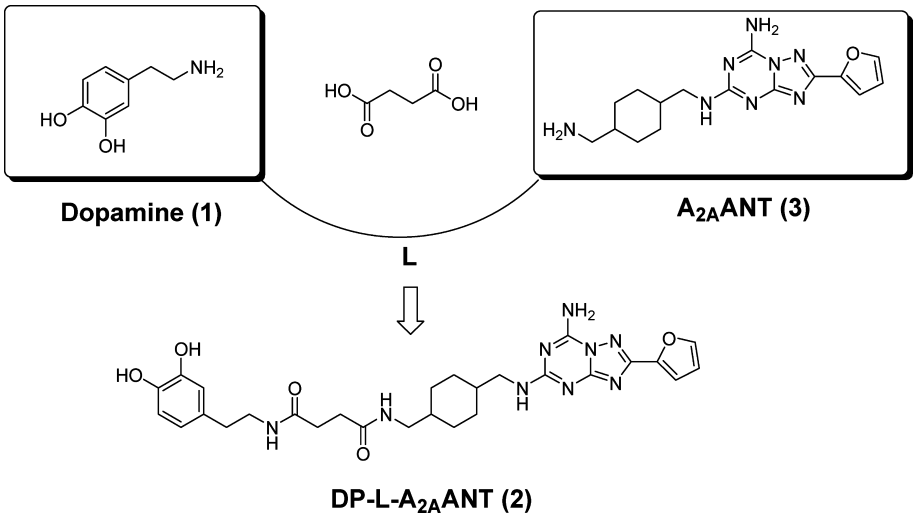

Figure 1. Design of the prodrug DP-L-A_{2A}ANT (**2**) obtained by the conjugation of dopamine (**1**) with a new A_{2A} antagonist (A_{2A}ANT, **3**) via a succinic linker (L).

treatment.[6] Other unwanted central effects related to L-DOPA therapy include hallucinations and mental confusion, due to stimulation of central dopaminergic receptors.[7] Finally, L-DOPA and dopamine would appear to be able to enhance neuronal degeneration by way of oxidative metabolism.[8] L-DOPA is, therefore, usually administered in association with other drugs, such as direct dopamine agonists or agents able to delay dopamine catabolism, with a view to reducing the L-DOPA dose required to act against the symptoms of PD. On the other hand, the drugs coadministered with L-DOPA are often not adequately effective in counteracting the parkinsonian motor disabilities and can induce unwanted effects which limit their therapeutic usage.[9] As a consequence, novel pharmacological approaches to the management of PD are required.

In the past decade increasing interest has been focused on A_{2A} adenosine receptors (ARs) as an important pharmacological target in PD. Indeed, preclinical and clinical studies have evidenced the ability of A_{2A} antagonists, such as istradefylline, to amplify the therapeutic effects of L-DOPA and reduce motor complications (wearing off, dyskinesias and on/off phenomena) deriving from its pulsatile long-term treatment.[10,11] It is known that A_{2A}ARs are coexpressed with dopaminergic D_2 receptors in striatopallidal GABA neurons, where they form heterodimeric complexes able to decrease the D_2 affinity for dopamine when the A_{2A}ARs are stimulated.[10,12,13] The A_{2A} antagonists can therefore enhance the therapeutic index of L-DOPA and D_2 agonists by blocking the A_{2A}ARs in these A_{2A}–D_2 heteromers.[13,14] Moreover, A_{2A} antagonists are able to reduce the L-DOPA induced dyskinesias by restoring the appropriate balance between A_{2A}ARs and D_2 receptors.[15−17] Finally, the neuroprotective effects of A_{2A} antagonists are considered as potentially useful in preventing the onset and development of PD.[18−20] The A_{2A} antagonists emerge, therefore, as a class of efficacious antiparkinsonian drugs for the future, whose coadministration with L-DOPA appears incisive for both the early stage and long-term treatment of PD. Formulations containing both L-DOPA or dopamine and A_{2A} antagonists should be developed with a view to enhancing the therapeutic action via the A_{2A}–D_2 heteromers.

In this paper we propose a new, potential antiparkinsonian compound DP-L-A_{2A}ANT (**2**) made up of dopamine (**1**) coupled via a succinic spacer to a new A_{2A} antagonist (Figure 1). The conjugation was obtained by amidic bonds. We chose, as A_{2A} antagonist, the 7-amino-5-(aminomethyl)-cyclohexylmethyl-amino-2-(2-furyl)-1,2,4-triazolo[1,5-*a*]-1,3,5-triazine trifluoroacetate A_{2A}ANT (**3**), a new triazolo-triazine derivative which does not have the limitation of aqueous solubility and is characterized by the presence of an amino group available for the coupling.[21] DP-L-A_{2A}ANT (**2**) was considered as a potential prodrug of both dopamine (**1**) and the A_{2A}ANT (**3**). The affinity of DP-L-A_{2A}ANT (**2**) and its potential hydrolysis products dopamine (**1**), dopamine-linker DP-L (**4**), A_{2A}ANT (**3**) and antagonist-linker L-A_{2A}ANT (**5**) were tested on human A_1, A_{2A}, A_{2B} and A_3ARs expressed in CHO cells. Binding experiments were also performed on A_{2A}ARs and D_2 receptors expressed in rat striatum. In this case the influence of the compounds on the dopamine affinity toward D_2 receptors in the presence and in the absence of the A_{2A} agonist CGS 21680 was also tested. Finally, we evaluated the hydrolysis pattern of the prodrug DP-L-A_{2A}ANT (**2**) in water, phosphate buffer, human whole blood and rat brain homogenates by monitoring the degradation and appearance over time of its hydrolysis products dopamine (**1**), DP-L (**4**), A_{2A}ANT (**3**) and L-A_{2A}ANT (**5**), whose stabilities were accurately determined according to the above incubation media.

MATERIALS AND METHODS

Materials. [^{3}H]-1,3-Dipropyl-8-cyclopentylxanthine ([^{3}H]-DPCPX, specific activity 120 Ci/mmol), [^{125}I]-4-aminobenzyl-5′-*N*-methylcarboxamidoadenosine ([^{125}I]-AB-MECA, specific activity 2000 Ci/mmol) and [^{3}H]-spiperone (specific activity, 16 Ci/mmol) were purchased from Perkin-Elmer Life and Analytical Sciences (Boston, MA, USA). [^{3}H]-4-(2-((7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-*a*][1,3,5]triazin-5-yl)amino)-ethyl)phenol ([^{3}H]-ZM 241385, specific activity 27 Ci/mmol) was obtained from American Radiolabeled Chemicals (St. Louis, MO, USA). [^{3}H]-cAMP, specific activity 21 Ci/mmol, was purchased from GE Healthcare (Chalfont St Giles, U.K.). Dopamine, forskolin, Ro 20-1724, DPCPX, R-phenylisopropyladenosine (R-PIA), ZM 241385 and heptafluorobutyric acid (HFBA) were obtained from Sigma (St. Louis, MO, USA). The dopamine-glutaric derivative (DP-glu, **6**) and the 5-amino-4-cyano-1-[3-(4-methoxyphenyl)propyl]pyrazole (AN-Pyr, **7**)

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were synthesized as previously described.[22,23] Methanol, acetonitrile and water were high-performance liquid chromatography (HPLC)-grade from Merck (Darmstadt, Germany). All other reagents were of analytical grade and obtained from commercial sources. Male Wistar rats were purchased from Harlan SRC (Milan, Italy).

Synthesis. Reactions were routinely monitored by thin-layer chromatography (TLC) on silica gel (precoated F_{254} Merck plates) and products visualized with iodine or potassium permanganate solution. Infrared spectra (IR) were measured on a Perkin-Elmer 257 instrument. ^{1}H NMR spectra were determined in $CDCl_3$ or DMSO-d_6 solutions with a Bruker AC 200 spectrometer, peak positions are given in parts per million (δ) downfield from tetramethylsilane as internal standard, and J values are given in hertz. Light petroleum ether refers to the fractions boiling at 40−60 °C. Melting points were determined on a Buchi-Tottoli instrument and are uncorrected. Chromatographies were performed using Merck 200−400 and 60−200 mesh silica gel. All reported products showed IR, ^{13}C NMR and ^{1}H NMR spectra in agreement with the assigned structures. Organic solutions were dried over anhydrous sodium sulfate. Elemental analyses were performed by the microanalytical laboratory of the Department of Chemistry, University of Trieste, and they were within ±0.4% of the theoretical values for C, H, and N.

Synthesis of 4-[[2-(3,4-Dihydroxyphenyl)ethyl]-amino]-4-oxobutanoic Acid (4). To a solution of dopamine hydrochloride (2.64 mmol) in dry pyridine (7.5 mL) was added succinic anhydride (3.2 mmol), and the mixture was stirred for 12 h at room temperature. After evaporation under reduced pressure, the crude reaction product was partitioned between ethyl acetate and water. The organic phase was dried, filtered and then evaporated under vacuum. The residue was purified by chromatography (CH_2Cl_2/MeOH 15:4) to afford the right compound as a colorless oil (70% yield). $[M + H]^+ = 254$. ^{1}H NMR (DMSO-d_6): 2.29 (m, 2H), 2.35−2.43 (m, 4H), 3.07−3.17 (m, 2H), 6.40 (dd, 1H), 6.54 (d, 1H, J = 2), 6.60 (d, 1H, J = 7.8), 7.86 (m, 1H). ^{13}C NMR (DMSO-d_6): 29.07, 29.32, 34.59, 40.55, 115.35, 115.81, 119.07, 130.12, 143.38, 144.91, 170.63, 173.77. IR (neat) cm^{-1}: 3220, 1685. Anal. ($C_{12}H_{15}NO_5$), C, H, N.

Synthesis of N1-((4-(((7-Amino-2-(furan-2-yl)-[1,2,4]-triazolo[1,5-*a*][1,3,5]triazin-5-yl)amino)methyl)-cyclohexyl)methyl)-N4-(3,4-dihydroxyphenethyl)-succinamide (2). A solution of 7-amino-5-(aminomethyl)-cyclohexylmethyl-amino-2-(2-furyl)-1,2,4-triazolo[1,5-*a*]-1,3,5-triazine trifluoroacetate (0.109 mmol) in DMF (2 mL) and triethylamine (0.109 mmol, 15.4 mL) was added dropwise to a solution of 4-[[2-(3,4-dihydroxyphenyl)ethyl]amino]-4-oxobutanoic acid (**4**, 0.132 mmol), HOBt (0.132 mmol), and WSC (0.132 mmol) in DMF (2 mL); the mixture was stirred at room temperature for 24 h. After the evaporation of the solvent under reduced pressure, the residue was purified by flash cromatography on NH_3 saturated silica gel (ethyl acetate/methanol 8:2) to furnish the compound as a colorless oil (80% yield). $[M + H]^+ = 578.3$. ^{1}H NMR (DMSO-d_6): 1.2−1.85 (m, 10H), 2.27−2.38 (m, 4H), 2.81−3.19 (m, 10H), 6.4 (dd, 1H, J = 2, J = 4), 6.52−6.75 (m, 3H), 7.1 (d, 1H, J = 4), 7.23 (bs, 1H), 7.56−7.88 (m, 3H), 8.07 (bs, 1H), 8.61 (s, 1H), 8.73 (s, 1H). IR (neat) cm^{-1}: 3480−3050, 1697, 1456. Anal. ($C_{28}H_{35}N_9O_5$), C, H, N.

Synthesis of 4-(((4-(((7-Amino-2-(furan-2-yl)-[1,2,4]-triazolo[1,5-*a*][1,3,5]triazin-5-yl)amino)methyl)-cyclohexyl)methyl)amino)-4-oxobutanoic Acid (5). A solution of 7-amino-5-(aminomethyl)cyclohexylmethyl-amino-2-(2-furyl)-1,2,4-triazolo[1,5-*a*]-1,3,5-triazine trifluoroacetate (**3**, 0.033 mmol) and succinic anhydride (0.039 mmol) in dry pyridine (0.5 mL) was stirred at room temperature for 24 h. Then, the solvent was removed under vacuum, and the residue was acidified to pH 5 with a 10% aqueous citric acid solution and extracted with ethyl acetate several times. The organic layer was dried, filtered and evaporated under reduced pressure to give the compound as a colorless foam (80% yield). $[M + H]^+ = 443$. ^{1}H NMR (CD_3OD): 1.45−1.91 (m, 10H), 2.45−2.61 (m, 4H), 2.99−3.31 (m, 6H), 6.59 (dd, 1H, J = 2, J = 4), 7.1 (bs, 1H), 7.51−7.55 (m, 1H), 7.68 (d, 1H, J = 2), 7.95−7.97 (m, 1H), 7.57 (d, 1H, J = 4). ^{13}C NMR (CD_3OD): 27.37, 29.82, 30.13, 30.38, 31.48, 36.41, 36.90, 39.06, 39.34, 44.27, 45.66, 112.78, 126.04, 145.76, 147.13, 149.02, 174.51, 176.23, 210.46. IR (neat) cm^{-1}: 3510−3050, 1685, 1470. Anal. ($C_{20}H_{26}N_8O_4$), C, H, N.

Membrane Preparation of CHO Cells Transfected with A_1, A_{2A}, A_{2B} or A_3ARs. The expression of the human A_1, A_{2A}, A_{2B} and A_3ARs in CHO cells has been previously described.[24] Briefly, for membrane preparation the cells were grown adherently and maintained in Dulbecco's modified Eagle's medium with nutrient mixture F12 (DMEM/F12) without nucleosides, containing 10% fetal calf serum, penicillin (100 U/mL), streptomycin (100 μg/mL), L-glutamine (2 mM) and Geneticin (G418, 0.2 mg/mL) at 37 °C in 5% CO_2/95% air. For membrane preparation the culture medium was removed and the cells were washed with phosphate buffer solution (PBS) and scraped off T75 flasks in ice-cold hypotonic buffer (5 mM Tris HCl, 2 mM EDTA, pH 7.4). The cell suspension was homogenized with Polytron (Kinematica Inc., Bohemia, NY, USA), the homogenate was spun for 10 min at 1000*g* and the supernatant was centrifuged for 30 min at 100000*g*. The membrane pellet was resuspended in 50 mM Tris HCl buffer, pH 7.4, containing 10 mM $MgCl_2$, incubated with 2 UI/mL of adenosine deaminase for 30 min at 37 °C and centrifuged again.

Preparation of Rat Striatum Membranes. Rat striatum was homogenized in 50 mM Tris HCl buffer, pH 7.4, with a Polytron and centrifuged for 20 min at 48000*g*.[25] To study A_{2A}ARs, the membrane pellet was resuspended in 50 mM Tris HCl buffer, pH 7.4, containing 10 mM $MgCl_2$ and incubated with 2 IU/mL adenosine deaminase for 30 min at 37 °C. Similar aliquots of membranes were suspended in 50 mM Tris HCl buffer, pH 7.4, with the aim to investigate D_2DRs.

Competition Binding Experiments in CHO Cells. The tested compounds were used in the range 1 nM to 1 μM in competition binding experiments in CHO cells transfected with human ARs. Briefly, hA_1CHO membranes (60 μg of protein/assay) and [^{3}H]-DPCPX (1 nM) as radioligand were incubated for 90 min at 25 °C.[24] Nonspecific binding was determined in the presence of DPCPX 1 μM. Competition binding experiments in hA_{2A}CHO membranes (60 μg of protein/assay) were carried out by using [^{3}H]-ZM 241385 (2 nM) as radioligand and were incubated for 60 min at 4 °C.[24] Nonspecific binding was determined in the presence of ZM 241385 1 μM. Competition binding experiments to hA_3ARs were conducted in membranes (80 μg of protein/assay) and [^{125}I]-AB-MECA (0.5 nM) at 37 °C for 60 min, and R-PIA 50 μM was used to evaluate the nonspecific binding.[24]

Competition Binding Experiments in Rat Striatum. The tested compounds, dopamine, ZM 241385 and butaclamol,

were used in the range 1 nM to 1 μM in competition binding experiments in rat striatum. Competition binding experiments to A_{2A}ARs (60 μg of protein/assay) were carried out by using [^{3}H]-ZM 241385 (2 nM) as radioligand and were incubated for 60 min at 4 °C.[24] Nonspecific binding was determined in the presence of ZM 241385 1 μM.

Competition binding experiments to D_2DRs (40 μg of protein/assay) were performed by incubating [^{3}H]-spiperone (1 nM) and rat striatum membranes for 15 min at 37 °C. The affinity of dopamine (1 nM to 1 μM) in the absence and in the presence of CGS 21680 (100 nM) was evaluated. Moreover, the effect of A_{2A}ANT **3**, L-A_{2A} ANT **5** and the prodrug DP-L-A_{2A} ANT **2** at 1 μM concentration on the affinity of dopamine was also investigated in the absence and in the presence of CGS 21680 (100 nM). Nonspecific binding was determined in the presence of butaclamol 1 μM.[25]

Saturation Binding Experiments in Rat Striatum. Saturation binding experiments to A_{2A}ARs in rat striatum (60 μg of protein/assay) were carried out by using [^{3}H]-ZM 241385 (0.01−20 nM) as radioligand and were incubated for 60 min at 4 °C.[25] Nonspecific binding was determined in the presence of ZM 241385 1 μM. Additional saturation binding experiments were performed in the absence and in the presence of dopamine (10 μM).

Saturation binding experiments to D_2 receptors in rat striatum aliquots (60 μg of protein/assay) were performed by using [^{3}H]-spiperone as radioligand.[25] The tissue membranes were incubated for 15 min at 37 °C with 8 to 10 concentrations of the radioligand [^{3}H]-spiperone (0.05−5 nM). Nonspecific binding was determined in the presence of butaclamol 1 μM. Additional saturation binding experiments were performed in the absence and in the presence of CGS 21680 (100 nM) or the tested compounds at the 1 μM concentration.

At the end of the incubation time in competition or saturation binding experiments, bound and free radioactivity were separated by filtering the assay mixture through Whatman GF/B glass fiber filters in Brandel cell harvester (Brandel, Unterföhring, Germany). Filter bound radioactivity was counted in a 2810 TR liquid scintillation counter Packard (Perkin-Elmer Life and Analytical Sciences, Boston, MA, USA).

Measurement of cAMP Levels. CHO cells transfected with the human A_{2B}ARs were suspended in a 0.5 mL incubation mixture containing NaCl 150 mM, KCl 2.7 mM, NaH_2PO_4 0.37 mM, $MgSO_4$ 1 mM, $CaCl_2$ 1 mM, glucose 5 mM, Hepes 100 mM, $MgCl_2$ 100 mM, pH 7.4 at 37 °C. Then 2.0 IU of adenosine deaminase/mL and 0.5 mM Ro 20-1724 as phosphodiesterase inhibitor were added and preincubated for 10 min in a shaking bath at 37 °C. The effect of the novel compounds at different concentrations (1 nM to 1 μM) was evaluated. The reaction was terminated by the addition of cold 6% trichloroacetic acid (TCA). The TCA suspension was centrifuged at 2000*g* for 10 min at 4 °C, and the supernatant was extracted four times with water saturated diethyl ether. The final aqueous solution was tested for cyclic AMP levels by a competition protein binding assay where samples of cyclic AMP standards (0−10 pmol) were added to each test tube containing trizma base 0.1 M, aminophylline 8.0 mM, mercaptoethanol 6.0 mM, pH 7.4 and [^{3}H]-cyclic AMP.[26] The binding protein, previously prepared from beef adrenals, was added to the samples and incubated at 4 °C for 150 min. At the end of the incubation time and after the addition of charcoal, the samples were centrifuged at 2000*g* for 10 min. The clear supernatant was mixed in Ultima Gold solution and counted in a 2810 TR liquid scintillation counter Packard (Perkin-Elmer Life and Analytical Sciences, Boston, MA, USA).

HPLC Analysis. The quantification of the prodrug DP-L-A_{2A}ANT (**2**) and its potential hydrolysis products dopamine (**1**), DP-L (**4**), A_{2A}ANT (**3**) and L-A_{2A}ANT (**5**) was performed by HPLC. The chromatographic apparatus consisted of a modular system (model LC-10 AD VD pump and model SPD-10A VP variable wavelength UV−vis detector; Shimadzu, Kyoto, Japan) and an injection valve with a 20 μL sample loop (model 7725; Rheodyne, IDEX, Torrance, CA). Separation was performed at room temperature on a reverse phase column (Phenomenex Synergi Polar RP 80 Å, 150 × 4.6 mm, 4 μm, ChemTek Analytica, Anfola Emilia, Bologna, Italy). Data acquisition and processing were carried out with a personal computer using Class-VP software (Shimadzu).

For dopamine (**1**) and DP-L (**4**) the detector was set at 280 nm. The mobile phase consisted of a mixture of 0.1% HFBA and methanol with a ratio of 85:15 (v/v). The flow rate was 0.8 mL/min. DP-glu (**6**) was employed as internal standard for plasma and brain homogenate samples (see below). The retention times of dopamine (**1**), DP-L (**4**) and DP-glu (**6**) were 6.3, 12.4, and 17.5 min, respectively. For the A_{2A}ANT (**3**) the detector was set at 260 nm. The mobile phase consisted of a mixture of 20 mM phosphate buffer (pH 2.5) and acetonitrile with a ratio of 85:15 (v/v). The flow rate was 0.8 mL/min. The DP-glu (**6**) was employed as internal standard for plasma and brain homogenate samples (see below). The retention times of A_{2A}ANT (**3**) and DP-glu (**6**) were 4.15 and 6.5 min, respectively.

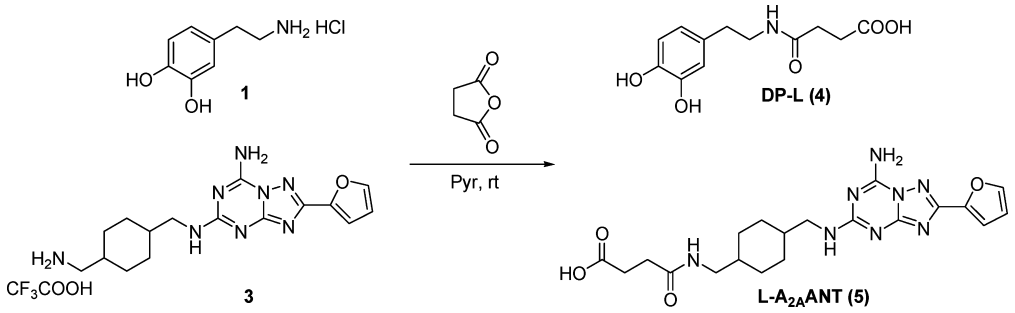
For the prodrug DP-L-A_{2A}ANT (**2**) and L-A_{2A}ANT (**5**) the detector was set at 248 nm. The mobile phase consisted of a mixture of water, methanol and acetonitrile with a ratio of 55:22:23 (v/v/v). The flow rate was 1 mL/min. AN-Pyr (**7**) was employed as internal standard for plasma and brain homogenate samples (see below). The retention times of L-A_{2A}ANT (**5**), DP-L-A_{2A}ANT (**2**), and AN-Pyr (**7**) were 4.15, 5.45, and 10.3 min, respectively.

The chromatographic precision for each compound was evaluated by repeated analysis (n = 6) of the same sample (25 μM). For all compounds dissolved in the aqueous phase, the calibration curves of peak areas versus concentration were generated in the range 0.5 to 100 μM.

Kinetic Analysis in Water and Phosphate Buffer. The prodrug DP-L-A_{2A}ANT (**2**) and its potential hydrolysis products dopamine (**1**), DP-L (**4**), A_{2A}ANT (**3**) and L-A_{2A}ANT (**5**) were incubated at 37 °C in water (HPLC grade, pH 6.0) or in 50 mM phosphate buffer (pH 7.4). Six milliliters of water or buffer were spiked with compound solutions resulting in a final concentration of 100 μM. At regular time intervals 200 μL samples were withdrawn and 10 μL aliquots were immediately injected into the HPLC apparatus. All the values were obtained as the mean of three independent experiments.

Kinetic Analysis in Whole Blood. The prodrug DP-L-A_{2A}ANT (**2**) and its potential hydrolysis products dopamine (**1**), DP-L (**4**), A_{2A}ANT (**3**) and L-A_{2A}ANT (**5**) were incubated at 37 °C in heparinized human whole blood obtained from healthy volunteers. Six milliliters of whole blood was spiked with compound solutions resulting in a final concentration of 100 μM. At regular time intervals, 400 μL samples were withdrawn and immediately centrifuged at 1500*g* at 4 °C for 15 min. 100 μL of the plasma obtained by centrifugation was quenched in 200 μL of ethanol (4 °C); 100 μL of internal

Scheme 1. Synthesis of Compounds DP-L (4) and L-A_{2A}ANT (5)



standard [100 μM DP-glu (**6**) for the analysis of dopamine (**1**) and DP-L (**4**); 500 μM DP-glu (**6**) for the analysis of A_{2A}ANT (**3**); 100 μM AN-Pyr (**7**) for the analysis of prodrug DP-L-A_{2A}ANT (**2**) and L-A_{2A}ANT (**5**)] was then added. After centrifugation at 13000*g* for 10 min, 300 μL aliquots were reduced to dryness under a nitrogen stream. Two hundred microliters of mobile phase was added and, after centrifugation, 10 μL was injected into the HPLC system. All the values were obtained as the mean of three independent incubation experiments.

The accuracy of the analytical method was determined by recovery experiments, comparing the peak areas extracted from blood test samples at 4 °C (n = 6) with those obtained by injection of an equivalent concentration of the analytes dissolved in their mobile phase. For all compounds analyzed, the calibration curves were constructed by employing eight different concentrations in whole blood at 4 °C ranging from 2 to 100 μM and expressed as peak area ratios of the compounds and their internal standard versus concentration.

Kinetic Analysis in Rat Brain Homogenates. The brains of male Wistar rats were immediately isolated after their decapitation, and homogenized in 5 volumes (w/v) of Tris HCl (50 mM, pH 7.4, 4 °C) with an ultra-Turrax (IKA Werke GmbH & Co. KG, Staufen, Germany) using 3 × 15 s bursts. The supernatant obtained after centrifugation (3000*g* for 15 min at 4 °C) was decanted off and stored at −80 °C before its employment for kinetic studies. The total protein concentration in the tissue homogenate was determined by using a Bio-Rad method,[27] with bovine albumin as reference standard, and resulted as 7.2 ± 0.4 μg of protein/μL.

The prodrug DP-L-A_{2A}ANT (**2**) and its potential hydrolysis products dopamine (**1**), DP-L (**4**), A_{2A}ANT (**3**) and L-A_{2A}ANT (**5**) were incubated at 37 °C in 3 mL rat brain homogenates, resulting in a final concentration of 100 μM. At regular time intervals, 100 μL samples were withdrawn and immediately quenched in 200 μL of ethanol (4 °C); 100 μL of internal standard (the same as described for blood samples) was then added. After centrifugation at 13000*g* for 10 min, 300 μL aliquots were reduced to dryness under a nitrogen stream. Two hundred microliters of mobile phase was added, and, after centrifugation, 10 μL was injected into the HPLC system. All the values were obtained as the mean of three independent incubation experiments.

The accuracy of the method and the calibration curves referred to rat brain homogenates were obtained as described for the blood samples.

Data and Statistical Analysis of Binding Experiments. The protein concentration of membrane employed for binding experiments was determined by the Bio-Rad method.[27] Dissociation equilibrium constants for saturation binding, affinity or K_D values, as well as the maximum densities of specific binding sites, B_{max}, were calculated for a system of one or two binding site populations by nonlinear curve fitting using the program Ligand (Kell Biosoft, Ferguson, MO) (Munson and Rodbard, 1980).[28] Functional experiments were calculated by nonlinear regression analysis using the equation for a sigmoid concentration−response curve (GraphPAD Prism, San Diego, CA).

Kinetic Calculations. The half-life values of the compounds showing a first order kinetic degradation were calculated from an exponential decay plot of its concentrations versus incubation time, using the computer program GraphPad Prism. The same software was employed for the linear regression of the corresponding semilogarithmic plots.

RESULTS

Synthesis. The desired compounds were synthesized as summarized in Schemes 1 and 2. The compound N1-((4-(((7-amino-2-(furan-2-yl)-[1,2,4]triazolo[1,5-*a*][1,3,5]triazin-5-yl)amino)methyl)cyclohexyl)methyl)-N4-(3,4-dihydroxyphenethyl)succinamide (**2**) was prepared following standard coupling conditions with hydroxybenzotriazole (HOBt) and *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide (WSC) in dimethylformamide (DMF), starting from compound 7-amino-5-(aminomethyl)cyclohexylmethyl-amino-2-(2-furyl)1,2,4-triazolo[1,5-*a*]-1,3,5-triazine trifluoroacetate (**3**) and 4-[[2-(3,4-dihydroxyphenyl)ethyl]amino]-4-oxobutanoic acid (**4**). Compound **3** was prepared as described in our previous work.[21] The derivatives DP-L (**4**) and 4-(((4-(((7-amino-2-(furan-2-yl)-[1,2,4]triazolo[1,5-*a*][1,3,5]triazin-5-yl)amino)-methyl)cyclohexyl)methyl)amino)-4-oxobutanoic acid (L-

Scheme 2. Synthesis of Compound DP-L-A_{2A}ANT (2)

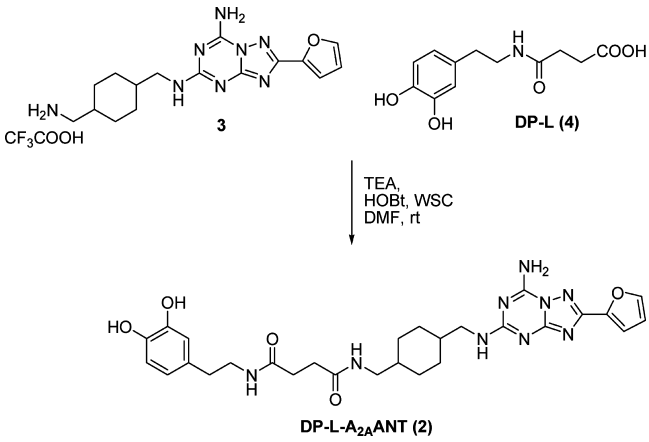


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Table 1. Affinity (K_i, nM) or Potency (IC_{50}, nM) Values of the Examined Compounds and Dopamine in CHO Membranes Transfected with hA_1, hA_{2A}, hA_{2B} and hA_3ARs and in Rat Striatum Membranes Expressing A_{2A}ARs and D_2 Receptors[a]

in vitro experiments	A_{2A}ANT (3)	L-A_{2A}ANT (5)	DP-L-A_{2A}ANT (2)	dopamine (1)	DP-L (4)
hA_1ARs ^{3}H-DPCPX binding (K_i, nM)	>5000	>5000	>5000	>10000	>5000
hA_{2A}ARs ^{3}H-ZM241385 binding (K_i, nM)	72 ± 5	35 ± 3	7.32 ± 0.65	>10000	>5000
hA_{2B}ARs cAMP assay (IC_{50}, nM)	>5000	>5000	>5000	>10000	>5000
hA_3ARs ^{125}I-AB-MECA binding (K_i, nM)	>5000	>5000	>5000	>10000	>5000
rat striatum A_{2A} ARs ^{3}H-ZM241385 binding (K_i, nM)	50 ± 2	24 ± 2	2.07 ± 0.23	>10000	>5000
rat striatum D_2 receptors ^{3}H-spiperone binding (K_i, nM)	>5000	>5000	>5000	2667 ± 120	>5000

[a]Data are expressed as mean (n = 4 experiments) ± SEM.

A_{2A}ANT (**5**)) were synthesized in high yield through the reaction of dopamine hydrochloride or compound **3** with succinic anhydride in dry pyridine. The DP-L was prepared as already described in the literature and further purified. Our spectroscopic data confirmed the structure, but they do not correspond to the data reported by Bonina et al.[29]

Affinity of Examined Compounds versus ARs. Competition binding experiments to human A_1, A_{2A} and A_3ARs, by using the examined compounds and dopamine in CHO cell membranes, were performed (Table 1). The novel compounds showed affinity values only for A_{2A}ARs revealing K_i values in the nanomolar range (Figure 2A). Additional competition

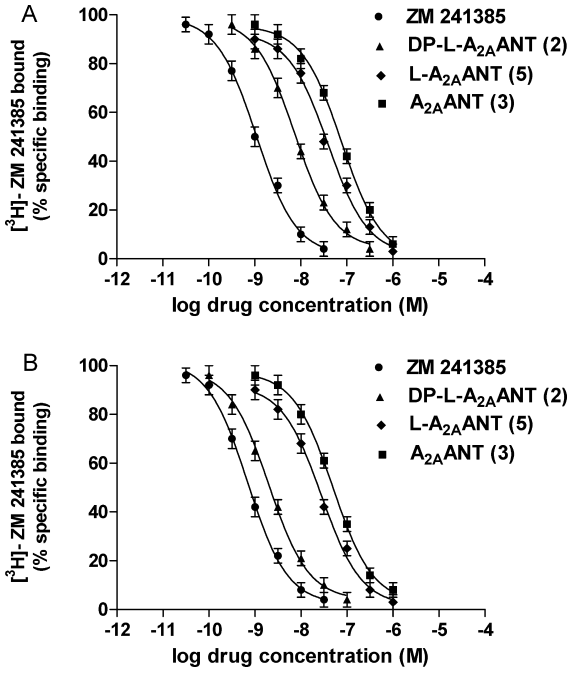

Figure 2. Affinity values of the examined compounds obtained by using competition binding experiments versus hA_{2A}ARs in CHO membranes (A) and in rat striatum membranes (B). Each value represents the mean ± SEM of four separate experiments performed in duplicate.

binding experiments were also performed in rat striatum where the compounds examined showed affinity values closely associated with those found in hA_{2A}CHO cells (Table 1, Figure 2B). Interestingly, the prodrug DP-L-A_{2A}ANT (**2**) showed a very high affinity versus A_{2A}ARs with a K_i of 2.07 ± 0.23 or 7.32 ± 0.65 nM in rat striatum or hA_{2A}CHO cells, respectively (Table 1, Figure 2). The affinity of a well-known A_{2A} antagonist ZM 241385 versus A_{2A}ARs was also investigated and showed a K_i of 0.67 ± 0.08 nM or of 0.85 ± 0.09 nM in rat striatum or hA_{2A}CHO cells, respectively. As expected, butaclamol was not able to interact with A_{2A}ARs.

Saturation binding experiments in hA_{2A}CHO cell membranes were performed to evaluate affinity (K_D) and receptor density (B_{max}) of the A_{2A}ARs in the absence and in the presence of DP 10 μM. Figure 3A illustrates saturation binding curves relative

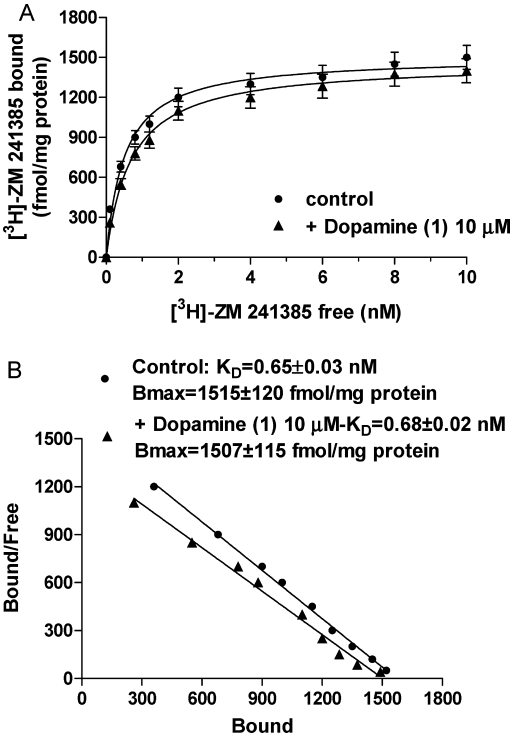

Figure 3. Saturation curves (A) and Scatchard plot (B) of [^{3}H]-ZM 241385 binding on A_{2A}ARs in rat striatum membranes. Each value represents the mean ± SEM of four separate experiments performed in duplicate.

to A_{2A}ARs showing affinity of 0.65 ± 0.03 nM and a very high receptor density of 1515 ± 120 fmol/mg protein. Similarly, in the presence of DP 10 μM the affinity of A_{2A}ARs was in the nanomolar range (K_i = 0.68 ± 0.02 nM) and the receptor density was 1507 ± 115 fmol/mg protein, suggesting that DP was not able to modify A_{2A} affinity or density. Scatchard plot analysis revealed the presence of a high affinity binding site as suggested by the linearity of the lines (Figure 3B). Computer analysis of the data failed to show a significantly better fit to a two site than to a one site binding model, indicating that in our experimental conditions one class of high affinity binding site is primarily present.

Affinity of Examined Compounds versus D_2 Receptors. Table 2 reports the results obtained by saturation and

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Table 2. (A) Affinity (K_D, nM) and Receptor Density (B_{max}, fmol/mg protein) of D_2 Receptors in Rat Striatum Membranes and (B) Affinity Values (K_i, nM) of the Examined Compounds on D_2 Receptors in the Absence and in the Presence of CGS 21680 (100 nM) in Rat Striatum Membranes[a]

(A) Affinity (K_D, nM) and Receptor Density (B_{max}, fmol/mg protein)	[^{3}H]-spiperone saturation binding experiments	
	K_D (nM)	B_{max} (fmol/mg protein)
control	0.25 ± 0.02	102 ± 8
+ CGS 21680 (100 nM)	0.23 ± 0.02	107 ± 9
+ A_{2A}ANT (**3**) (1 μM)	0.24 ± 0.02	101 ± 9
+ L-A_{2A}ANT (**5**) (1 μM)	0.25 ± 0.01	103 ± 8
+ DP-L-A_{2A}ANT (**2**) (1 μM)	0.23 ± 0.02	100 ± 7
(B) Affinity Values (K_i, nM)	**[^{3}H]-spiperone competition binding experiments**	
	K_i (nM)	K_i (nM) + CGS 21680 (100 nM)
dopamine	2667 ± 120	14433 ± 233
+ A_{2A}ANT (**3**) (1 μM)	2650 ± 104	2767 ± 117
+ L-A_{2A}ANT (**5**) (1 μM)	2700 ± 153	2623 ± 101
+ DP-L-A_{2A}ANT (**2**) (1 μM)	2633 ± 174	2730 ± 114

[a]Data are expressed as mean (n = 4 experiments) ± SEM.

competition binding experiments versus D_2 receptors in rat striatum. Figure 4 shows D_2 receptor binding characteristics as affinity and density in rat striatum in the absence and in the presence of CGS 21680 (100 nM). The well-known A_{2A} agonist was not able to modify K_D or B_{max} values as reported in Figure 4A and 4B. The compounds examined at the 1 μM concentration did not modify binding parameters of [^{3}H]-spiperone in rat striatum (Table 2A). Competition binding experiments to D_2 receptors were also performed in rat striatum with the aim to evaluate affinity values of DP (K_i = 2667 ± 120 nM) and butaclamol (K_i = 3.93 ± 0.12 nM). In contrast ZM 241385, a well-known A_{2A} antagonist, did not interact with D_2 receptors expressing in rat striatum (K_i > 1000 nM). In addition, no affinity values were found for the novel compounds versus D_2 receptors (Table 1). The affinity value of dopamine (**1**) was evaluated in the presence of CGS 21680 showing a significant increase in the K_i value to 14433 nM, corresponding to reduced affinity (Table 2B, Figure 5).

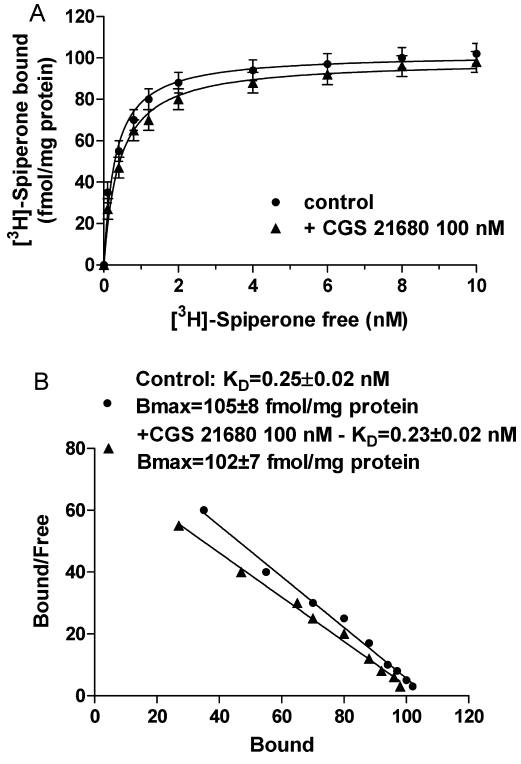

Figure 4. Saturation curves (A) and Scatchard plot (B) of [^{3}H]-spiperone binding on D_2 receptors in rat striatum membranes. Each value represents the mean ± SEM of four separate experiments performed in duplicate.

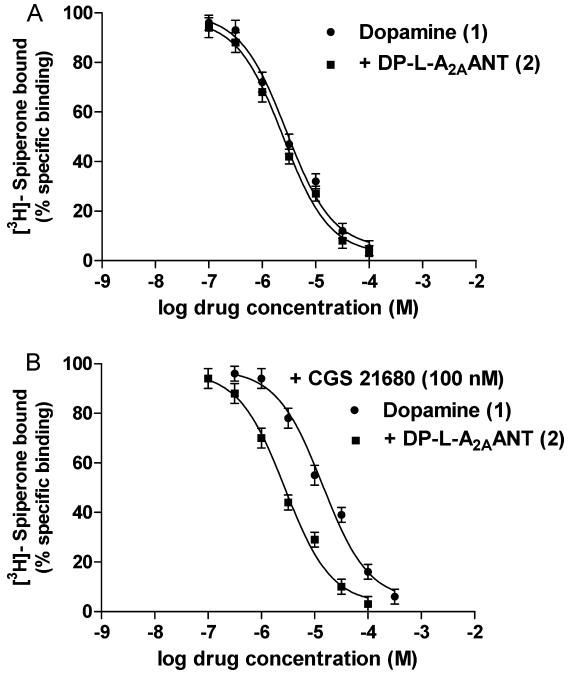

Figure 5. Affinity value of dopamine (**1**) in the absence and in the presence of DP-L-A_{2A}ANT2 by using competition binding experiments versus D_2 receptors in rat striatum membranes. The affinity values were calculated both in the absence (A) or in the presence (B) of CGS 21680 (100 nM). Each value represents the mean ± SEM of four separate experiments performed in duplicate.

Interestingly, the compounds examined at the 1 μM concentration blocked the effect of CGS 21680 restoring the affinity of dopamine (**1**) to control values (Table 2B, Figure 5).

HPLC and Stability Studies. The aim of our work was to evaluate the potential hydrolysis pattern of the prodrug DP-L-A_{2A}ANT (**2**) in different media such as water, phosphate buffer, human whole blood and rat brain homogenates. For this purpose it was necessary to detect and quantify, in all incubation media, not only the prodrug but also its potential hydrolysis products dopamine (**1**), DP-L (**4**), A_{2A}ANT (**3**) and L-A_{2A}ANT (**5**). In order to do so, an efficacious analytical method was developed based on the employment of a reverse phase HPLC column, characterized by a polar ether-linked phenyl phase, able to provide a satisfactory polar and aromatic reversed phase selectivity of the compounds analyzed by us. In particular, the analytical HPLC method for dopamine (**1**) and

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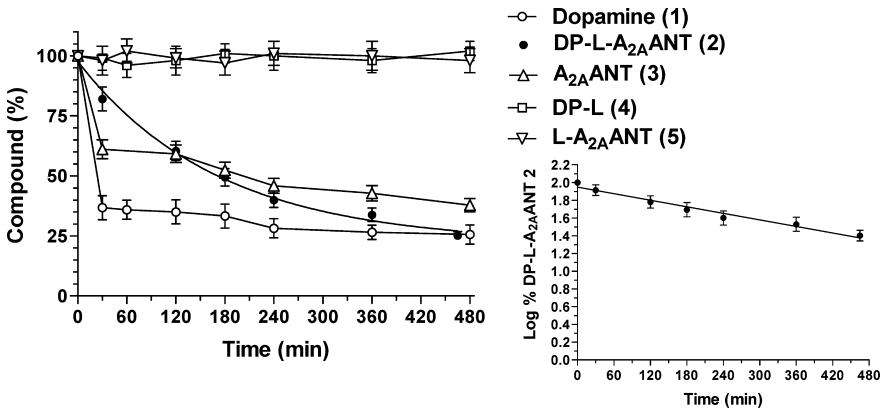

Figure 6. Degradation profile of the prodrug DP-L-A_{2A}ANT (**2**) and its potential hydrolysis products in human whole blood. The inset shows the semilogaritmic plot of the prodrug profile; its linearity (n = 7, r = 0.985, P < 0.0001) provides evidence of a degradation following apparent first order kinetics (half-life = 2.73 ± 0.23 h). Data are reported as the mean ± SD of three independent experiments.

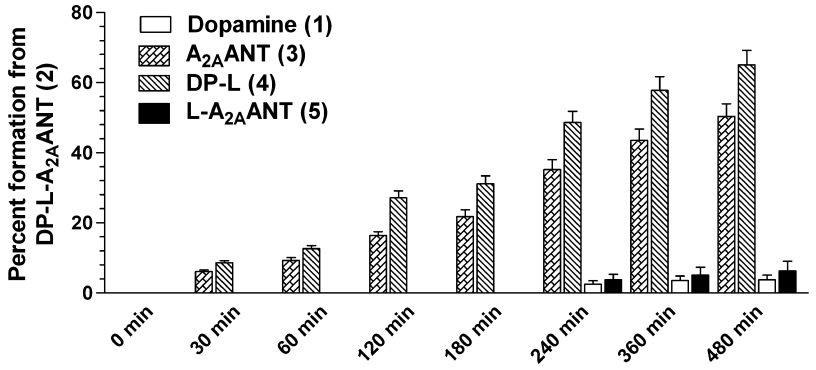

Figure 7. Appearance in whole blood of the compounds derived by the hydrolysis of the prodrug DP-L-A_{2A}ANT (**2**) during its incubation at 37 °C. The values are reported as the percentage of the overall amount of incubated prodrug. Data are reported as the mean ± SD of three independent experiments.

its derivative DP-L (**4**) was optimized with a mobile phase containing heptafluorobutyric acid, allowing their satisfactory retention at 15% (v/v) methanol concentration in the mobile phase.

The chromatographic precision for all the compounds dissolved in aqueous phase and their internal standards were represented by the relative standard deviation (RSD) values ranging, among the different molecules, between 0.87% and 1.36%. The calibration curves of the compounds incubated in aqueous phase were linear over the range of 0.5−100 μM (n = 8, r > 0.998, P < 0.0001). The average recoveries ± SD of the compounds from human whole blood and rat brain homogenates ranged between 61.2 ± 2.9% and 97 ± 4.8%. The concentrations of the prodrug DP-L-A_{2A}ANT (**2**) and its potential hydrolysis products were therefore referred to as peak area ratio with respect to their internal standards. The precision of the method based on peak area ratio was represented by RSD values ranging between 1.2% and 1.6% among the analyzed molecules. The calibration curves referred to the compounds incubated in human whole blood and rat brain homogenates were linear over the range 2−100 uM (n = 8, r > 0.996, P < 0.0001). No interferences were observed from plasma or brain homogenate extract components.

The prodrug DP-L-A_{2A}ANT **2** and its potential hydrolysis products dopamine (**1**), DP-L (**4**), A_{2A}ANT (**3**) and L-A_{2A}ANT (**5**) were not degraded in water during incubation for eight hours at 37 °C. Similarly, the compounds were not degraded in phosphate buffer (pH 7.4) with the exception of dopamine (**1**), which decomposed following apparent first order kinetics (data not shown) with a half-life of 6.3 ± 0.4 h. Dopamine (**1**) incubated in human whole blood was degraded following a biphasic pattern, which was relatively rapid in the first stage (more than 60% of the drug degraded in 30 min) and slower in the second phase, where about 11% of incubated drug was degraded within eight hours (Figure 6). The A_{2A}ANT (**3**) incubated in whole blood showed a degradation pattern similar to that of dopamine (**1**), even if the rates were different: in particular, about 40% of incubated antagonist was degraded in 30 min, and then a further decrease of about 25% was registered within eight hours (Figure 6). High stability was, instead, registered in human whole blood for the derivatives DP-L (**4**) and L-A_{2A}ANT (**5**), whose amounts had not decreased during eight hours of incubation at 37 °C (Figure 6). Finally, the prodrug DP-L-A_{2A}ANT (**2**) appeared degraded in human whole blood following pseudo first order kinetics (Figure 6, half-life = 2.73 ± 0.23 h), as confirmed by the linear pattern of the semilogarithmic plot (n = 7, r = 0.985, P < 0.0001) reported in the inset of Figure 6, and suggesting, therefore, a prodrug degradation governed by hydrolysis processes. In order to verify this hypothesis, the blood samples related to DP-L-A_{2A}ANT (**2**) incubation were also analyzed for the quantification of its potential hydrolysis products dopamine (**1**), DP-L (**4**), A_{2A}ANT (**3**) and L-A_{2A}ANT (**5**). The results of this investigation are shown in Figure 7, where the appearance over time of the hydrolysis products is reported as a percentage of the overall amount of prodrug incubated in whole blood. In

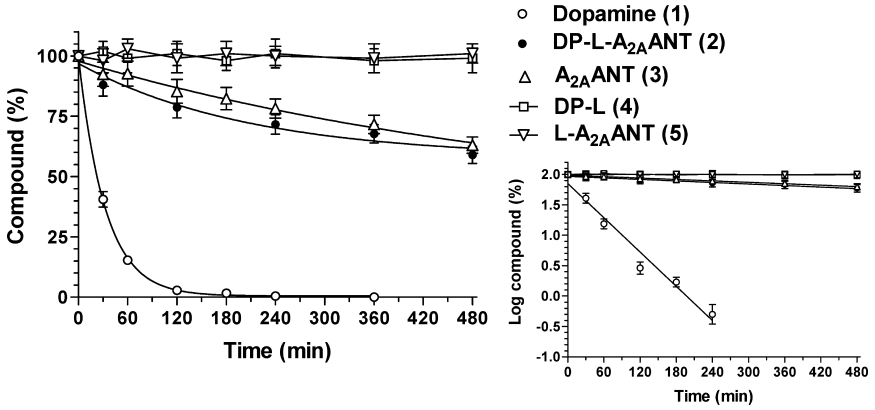

Figure 8. Degradation profile of the prodrug DP-L-A_{2A}ANT (**2**) and its potential hydrolysis products in rat brain homogenates. The inset shows the semilogaritmic plot of the compounds; their linearity ($n \geq 6$, $r > 0.970$, $P \leq 0.001$) evidences a degradation of dopamine (**1**), A_{2A}ANT (**3**) and prodrug DP-L-A_{2A}ANT (**2**), following apparent first order kinetics (half-life = 22.5 ± 1.5 min for dopamine (**1**), higher than eight hours for A_{2A}ANT (**3**) and prodrug DP-L-A_{2A}ANT (**2**)). Data are reported as the mean ± SD of three independent experiments.

particular, it can be observed that the amounts of A_{2A}ANT (**3**) and DP-L (**4**), delivered over time, are relatively high, since their values are near to those derived from the degradation pattern of the prodrug. Indeed, the amounts of A_{2A}ANT (**3**) and DP-L (**4**) delivered within eight hours corresponded to about 50% and 65%, respectively, of the incubated prodrug DP-L-A_{2A}ANT (**2**), with respect to its 75% degradation. Figure 7 also provides evidence that, after 4 h of incubation, dopamine (**1**) and L-A_{2A}ANT (**5**) became detectable in whole blood, and that after eight hours their amounts corresponded to 4 and 6%, respectively, of the incubated prodrug. It is important to underline that DP-L (**4**) and L-A_{2A}ANT (**5**) were characterized by their inability to be degraded in blood (Figure 6) and that the sum of their amounts delivered during the prodrug DP-L-A_{2A}ANT (**2**) incubation in whole blood appeared compatible with its degradation pattern. Our results indicate, therefore, that the prodrug degradation in human blood is mainly governed by the hydrolysis of the N1 amide group obtained by amidation of the A_{2A}ANT (**3**) with the linker and weakly governed by the hydrolysis of N4 amide group obtained by amidation of dopamine (**1**) with the linker.

The potential ability of the prodrug DP-L-A_{2A}ANT (**2**) to be hydrolyzed in central nervous system (CNS) environments was investigated by incubating it in rat brain homogenates. Also in this case, not only the prodrug but also its potential hydrolysis products were detected. As reported in Figure 8, dopamine (**1**) showed a relatively fast degradation in rat brain homogenates, following pseudo first order kinetics with a half-life of 22.5 ± 1.5 min. The A_{2A}ANT (**3**) and the prodrug DP-L-A_{2A}ANT (**2**) also appeared degraded following pseudo first order kinetics, even if slower than dopamine (**1**) (Figure 8), and showed half-life values higher than eight hours. In particular, after eight hours of incubation, the degradations of the prodrug DP-L-A_{2A}ANT (**2**) and A_{2A}ANT (**3**) were 41% and 37%, respectively. The pseudo first order kinetics related to the degradation of the three compounds was confirmed by the linearity of the semilogarithmic plots ($n \geq 6$, $r > 0.970$, $P \leq 0.001$) reported in the inset of Figure 8. As found in human whole blood, the derivatives DP-L (**4**) and L-A_{2A}ANT (**5**) showed high stability in rat brain homogenates, since their amounts did not decrease during eight hours of incubation at 37 °C (Figure 8).

In order to verify whether the degradation of the prodrug DP-L-A_{2A}ANT (**2**) in rat brain homogenates was related to hydrolysis processes, the samples obtained from its incubation were also analyzed for the quantification of its hydrolysis product. The results of this investigation are reported in Figure 9, where the appearance over time of the hydrolysis products is

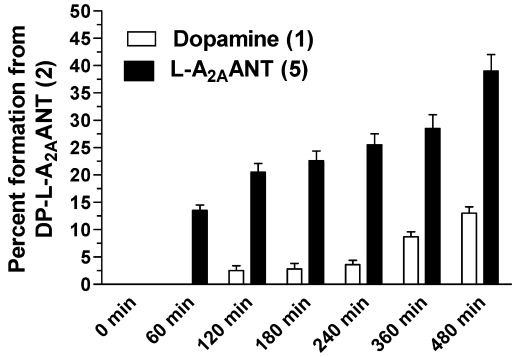

Figure 9. Appearance of rat brain homogenates of the compounds derived by the hydrolysis of the prodrug DP-L-A_{2A}ANT (**2**) during its incubation at 37 °C. The values are reported as the percentage of the overall amount of incubated prodrug. Data are reported as the mean ± SD of three independent experiments.

reported as a percentage of the overall amount of prodrug incubated in rat brain homogenates. Neither the A_{2A}ANT (**3**) nor the derivative DP-L (**4**) was detected within eight hours incubation of the prodrug DP-L-A_{2A}ANT (**2**). On the other hand, L-A_{2A}ANT (**5**) and dopamine (**1**) appeared detectable after one and two hours of incubation, respectively, and after eight hours their amounts delivered appeared to be 13% and 39% of the incubated prodrug. It is interesting to observe that the amounts of the delivered L-A_{2A}ANT (**5**), that was not degraded in rat brain homogenates, appeared compatible with the prodrug DP-L-A_{2A}ANT (**2**) degradation pattern. On the other hand, the amounts of dopamine (**1**) were lower, according to its relatively high instability in rat brain homogenates. Our results indicate, therefore, that the prodrug degradation in rat brain homogenates is governed exclusively by the hydrolysis of the amide group obtained from the amidation of the dopamine (**1**) with the linker. This process allowed us to obtain a controlled and prolonged release of dopamine (**1**): a comparison of Figure 8 with Figure 9 evidences that relatively high amounts of free dopamine were totally degraded within 3

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h, whereas the prodrug appeared able to perform a prolonged delivery of small DP amounts for at least eight hours.

DISCUSSION

Adenosine antagonist and dopamine interactions have been widely reported in CNS in both behavioral and biochemical studies.[30] In vitro and in vivo developments of selective A_{2A} antagonists, like istradefylline or preladenant, have yielded the greatest improvement in the mobility of PD patients by simultaneous activation of D_2 receptors and inhibition of A_{2A}ARs.[31−34] On the other hand, it has been observed that the administration of A_{2A}AR antagonists in monotherapy does not improve motor symptoms in PD, suggesting a need for dopamine receptor activation.[35,36] It has also been postulated that the interaction between A_{2A}ARs and D_2 receptors may involve the formation of heteromeric complexes.[37] Various studies are present in the literature showing that A_{2A} antagonists exert marked efficacy on locomotor activity in various models of PD.[38] In a well-known in vitro model, widely used for PD studies represented by PC 12 cells, treatment with adenosine agonists mediated a reduction in D_2 receptors and a decrease in the dopamine affinity.[14] In addition, in PC 12 cells the A_{2A} antagonists, inhibiting dopamine uptake, increased the concentrations of dopamine available to interact with D_2 receptors and a consequent marked activation of dopaminergic pathway providing a significant improvement as far as symptoms are concerned.[25] Moreover, it has been demonstrated that A_{2A}ARs are upregulated in PD patients in comparison with healthy subjects.[25] Thus, A_{2A} antagonists might block the A_{2A}ARs by reducing the activity in the indirect pathway known to produce motor inhibition.[39] On the other hand, it has been reported that overstimulation of A_{2A}ARs could be due to the uncontrolled discharge of corticostriatal terminals leading to the increase in adenosine levels through the ATP catabolic degradation.[40] It is well-known that the activation of D_2 leads to endocannabinoid production that is able to activate presynaptic CB_1 receptors at corticostriatal terminals. Since presynaptic A_{2A}AR activation inhibits CB_1 receptors in the striatum, it prevents the CB_1-mediated inhibition of motor activity and of depolarization-induced glutamate release.[41] Postsynaptically, A_{2A}AR stimulation inhibits the endocannabinoid production mediated by D_2 activation.[42] Thus, A_{2A}AR and D_2 receptors might act in concert to regulate endocannabinoid function in the striatum. As a consequence, the coadministration of A_{2A} antagonists with L-DOPA or D_2 agonists appears efficacious in preventing the motor complications, such as dyskinesias, induced by pulsatile long-term treatment of dopaminergic drugs. In this case the A_{2A} antagonists most likely contribute to restoring the appropriate balance between A_{2A} ARs and D_2 receptors.[11,15−17] It could be of interest to note that patients with advanced PD treated with levodopa/carbidopa, formulated as intestinal gel (DUODOPA) to avoid pulsatile L-DOPA administration, showed evident improvements in motor fluctuations and dyskinesias.[43−45] Ongoing studies are looking into potential future therapies, including medications that provide both monoaminooxidase B and glutamate inhibition, a sustained-release levodopa prodrug, a carbidopa subcutaneous patch, an oral neurotrophic factor inducer and different antidyskiniesia medications.[36,46−50] Nowadays, there is an urgent need for other therapies that provide anti-Parkinsonian benefits but which, at the same time, avoid dyskinesia, and reduce the progression of the pathology.

At present, the relevance of the A_{2A}−D_2 heteromeric complexes in PD pathophysiology and treatment is widely investigated, suggesting that these heteromers remain in the dorsal striatopallidal GABA pathway after degeneration of the nigrostriatal pathway.[38] Thus, the supersensitive D_2 receptors remain under strong antagonistic A_{2A}ARs control.[51] As a consequence, the DP/adenosine bivalent action could represent a novel concept in PD pharmacotherapy.

The idea of building a molecular conjugate able to interact in a dual synergic way on different biological targets is quite common in medical chemistry. Novel compounds characterized by the presence of A_{2A} antagonist and dopaminergic pharmacophores that, either directly or after their release, can perform the bivalent action of A_{2A}AR blockade and D_2 receptor activation could constitute highly interesting drug candidates. To this end, we decided to conjugate an A_{2A} antagonist and the dopamine, the natural ligand of dopaminergic receptor, through the linkage with an opportune spacer. We chose to link the A_{2A} antagonist and the dopamine to the spacer through amidic bonds since these moieties can be cleaved by endogenous enzymes, with consequent release of the two drugs, even if they are slightly more resistant than ester functions. This approach can potentially induce a controlled release of dopamine, and may, therefore, considerably limit its typical pulsatile pattern in the brain that generally follows L-DOPA administration. Moderate and nonpulsating tones of dopamine in the CNS can therefore limit its neuronal toxicity and avoid the long-term side effects derived from pulsatile treatments. Succinic acid seemed the best candidate as a spacer given the high reactivity of its anhydride and its good toxicological tolerability, if released into the bloodstream. Our research team has been working toward the development of various structures such as A_{2A} antagonists.[51] Recently, we synthesized several triazolotriazine derivatives endowed with a good affinity and selectivity for A_{2A}ARs.[21] Among these new structures, the 7-amino-5-(aminomethyl)cyclohexylmethyl-amino-2-(2-furyl)-1,2,4-triazolo[1,5-*a*]-1,3,5-triazine trifluoroacetate (**3**) was selected as a better analogue to be conjugated through its amine group on the side chain to the succinic acid. Compound **3** has a good pharmacological profile with a high affinity for the receptor (hA_{2A} 72 nM) and very high selectivity versus all the other human A_1, A_{2B}, and A_3ARs. The presence of the amine group on the side chain allowed us to link the succinic acid without influencing the other parts of the molecule that are necessary to the receptor binding, such as the amino group at position 7.[21] Moreover, in accordance with our previous experience, we hypothesized that a steric hindrance at the side chain could induce an affinity improvement.[52]

The prodrug DP-L-A_{2A}ANT (**2**) was not able to interact toward D_2 receptors, but it did show high affinity toward A_{2A}ARs, with K_i values of 2.07 or 7.32 nM in rat striatum or hA_{2A}CHO cells, respectively, 1 order of magnitude lower than those of the A_{2A}ANT (**3**). Moreover, the L-A_{2A}ANT (**5**), potentially derived from hydrolysis of DP-L-A_{2A}ANT (**2**), showed affinity values toward A_{2A}ARs similar to those of the A_{2A}ANT (**3**). The latter compound and its derivatives **2** and **5** appeared, moreover, highly selective for A_{2A}ARs and unable to interact with the D_2 receptors. These results were predictable since it is known, from docking studies, that the side chain protrudes from the binding cavity and hydrophilic substitutions can interact with the hydrophilic portion of the extracellular loops.[21] The compounds examined were not able to modify the affinity and potency studied by means of ^{3}H-spiperone

saturation binding experiments versus D_2 receptors, suggesting that their presence did not change the kinetic parameters of these receptors. Interestingly, the compounds tested at the 1 μM concentration blocked the reduction in the dopamine affinity by CGS 21680, restoring the affinity of dopamine to control values.

Recently, a novel designed synthesized family of heterobivalent ligands, containing a D_2 agonist and an A_{2A} antagonist acting as a probe for A_{2A}–D_2 heteromers, has been studied. These data suggest the cooperative effect derived from the simultaneous interaction of heterobivalent ligands with receptors in brain striatum, but not in cotransfected cells with the single homomers, thus evidencing the presence of the A_{2A}–D_2 heteromers in striatum tissue.[53]

With the aim to evaluate the hydrolysis pattern of the prodrug DP-L-A_{2A}ANT (**2**) in physiologic environments (and thus its potential bivalent action on A_{2A}–D_2 heteromers obtained by the release of dopamine (**1**)), we first compared the stabilities of the prodrug **2** and its potential hydrolysis products (dopamine (**1**), DP-L (**4**), A_{2A}ANT (**3**) and L-A_{2A}ANT (**5**)) in water, phosphate buffer, human whole blood and rat brain homogenates; then, over time, we detected the appearance of the hydrolysis products in the fluids where the prodrug **2** appeared degraded. All the compounds analyzed were not found to be degraded in water, whereas in phosphate buffer only dopamine (**1**) showed degradation over time. This result confirms previous studies on dopamine (**1**) stability[54] and suggests that its chemical degradation, probably oxidative,[55] is influenced by the pH and/or ionic strength of the incubation medium. Dopamine was also degraded in human whole blood, in conformity with data in the literature reporting a severe degradation of dopamine (**1**) the day after its incubation at 37 °C in human plasma.[55] The A_{2A}ANT (**3**) showed a biphasic degradation pattern over time in whole blood, suggesting its poor stability at peripheral level in the body. The prodrug DP-L-A_{2A}ANT (**2**) was degraded in whole blood following pseudo first order kinetics. We have demonstrated that this degradation is related to a hydrolysis process which is mainly related to the amide group coupling the A_{2A}ANT (**3**) with the linker, and very poorly to the amide group coupling dopamine (**1**). These data suggest that, at the peripheral level, the prodrug could release limited amounts of dopamine, thus avoiding induction of its typical peripheral side effects. The release process from the prodrug DP-L-A_{2A}ANT (**2**) of the A_{2A}ANT (**3**) could, moreover, contribute to increasing its half-life. The other main hydrolysis product of prodrug (**2**), the DP-L (**4**), was totally inactive toward the dopaminergic receptors. Surprisingly, this compound and its homologous L-A_{2A}ANT (**5**) were not degraded in human whole blood. This behavior, apparently paradoxical if we take into account the hydrolysis pattern of the prodrug (**2**), may be attributed to the presence of the carboxylic groups belonging to these compounds, that, at physiologic pH, are negatively charged. The presence of this charge can inhibit the enzymatic hydrolysis activity on the amide groups. A similar phenomenon has been demonstrated in the case of aspirin, which, in plasma, is normally not hydrolyzed to salicylic acid. On the other hand, the esterification of the carboxylic acid group of aspirin renders its *O*-acetyl ester highly susceptible to plasma-mediated hydrolysis.[56,57] This phenomenon is due to butyrylcholinesterase, a dominant esterase in human plasma that is not able to induce the hydrolysis of negatively charged substrates, but is extremely efficient in processing neutral molecules.[58] The linker-coupled compounds **4** and **5** showed high stability also in rat brain homogenates, unlike the other compounds analyzed, dopamine (**1**), the A_{2A}ANT (**3**) and the prodrug DP-L-A_{2A}ANT (**2**) that were all degraded following pseudo first order kinetics. The degradation rate of dopamine appeared relatively fast, considering that the total amounts incubated disappeared within 3 h. These data reflect the metabolic processes that involve dopamine in CNS,[59] where its degradation can be a source of free radicals.[60] Concerning these metabolic processes, it has been hypothesized that the high dopamine concentrations induced in the brain by L-DOPA therapy may potentially contribute to the progression of oxidative damage of dopaminergic neurons.[59,61−63] We have demonstrated that in rat brain homogenates the prodrug **2** is hydrolyzed exclusively on the amidic group coupling dopamine (**1**), allowing its controlled release. These data suggest that, in the CNS, the prodrug **2** may allow us to obtain a moderate dopamine tone for higher interval times than those derived from L-DOPA therapy. This behavior may be useful in limiting not only the neuronal toxicity caused by the oxidative metabolism of dopamine but also the side effects derived from pulsatile L-DOPA long-term treatments, indicating the prodrug **2** as a potential alternative to the DUODOPA systems. These beneficial effects may be corroborated by the A_{2A} antagonistic activity of the prodrug **2** itself and its main hydrolysis product, the L-A_{2A}ANT (**5**), whose stability in rat brain homogenates has been shown to be higher than that of its parent antagonist (**3**). Indeed, the neuroprotective effects of A_{2A} antagonists on CNS and their ability to potentiate the therapeutic effects of dopamine and limit the motor complications derived from long-term L-DOPA treatment are well-known.[10,15,16,18−20]

Several prodrugs able to release dopamine by enzymatic hydrolysis have been found to induce antiparkinsonian effects on rats,[29,64−66] suggesting the existence, in the brain, of enzymes able to obtain the release of dopamine at extracellular level. These phenomena allow us to hypothesize that the release of dopamine observed in brain homogenates may involve extracellular enzymatic systems.

Taking into account the aspects described above, the prodrug DP-L-A_{2A}ANT (**2**) appears to be a good candidate for nasal administration, a promising way for the cerebral uptake of potent neuroactive agents.[67,68] We have demonstrated that microparticulate formulations based on chitosan can be useful in promoting the CNS entry of neuroactive drugs via the nasal pathway.[69,70] These new results may help guide and improve future pharmacological treatment in PD, even if further studies are necessary to better investigate the *in vivo* administration and effects of the prodrug **2**.

■ CONCLUSIONS

The prodrug DP-L-A_{2A}ANT (**2**) was designed with a view to conjugating the beneficial effects against PD obtained by a combined action of dopamine and A_{2A} antagonists in CNS. This action is focused on striatal A_{2A}–D_2 heteromers. The prodrug **2**, able to act as a potent and selective A_{2A} antagonist, does not release dopamine in human whole blood by hydrolysis, most likely reducing the probability of inducing the typical dopaminergic side effects at the peripheral level. In rat brain homogenates the prodrug **2** is able to control the release of dopamine and, therefore, to increase its poor stability. Both the prodrug and hydrolysis product L-A_{2A}ANT (**5**), the latter characterized by high stability in physiologic media, are able to increase the dopamine affinity toward striatal D_2

receptors by counteracting the activity of A_{2A} agonists. These combined effects suggest the prodrug approach as promising for both early and long-term pharmacological treatment of PD.

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Notes

The authors declare no competing financial interest.

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