

Synthesis and Study of 5'-Ester Prodrugs of N⁶-Cyclopentyladenosine, a Selective A₁ Receptor Agonist

Alessandro Dalpiaz,¹ Angelo Scatturin,¹
Enea Menegatti,¹ Fabrizio Bortolotti,¹
Barbara Pavan,² Carla Biondi,² Elisa Durini,¹ and
Stefano Manfredini^{1,3}

Received December 10, 2000; accepted January 8, 2001

Purpose. A series of 5'-esters of N⁶-cyclopentyladenosine (CPA) were prepared with the aim to improve stability and bioavailability of selective A₁ agonists. Log P values, stability, affinity, and activity toward human adenosine A₁ receptors were evaluated.

Methods. An appropriate synthetic procedure was adopted to avoid concomitant deamination at position 6. Log P values were obtained by the Mixxor system. The stability of CPA and its 5'-ester was evaluated in human plasma and whole blood and analyzed with high-performance liquid chromatography. The affinities to human A₁ receptor expressed by N⁶-cyclohexyladenosine cells were obtained by binding experiments. The activities were evaluated by measurements of the inhibition of forskolin stimulated 3'-5'-cyclic adenosine monophosphate, performing competitive binding assays.

Results. All prodrugs were more lipophilic than CPA, and their hydrolysis, in whole blood and in plasma, was found related, respectively, to the length and hindrance of 5'-substituents. Affinity and activity values indicated a very weak interaction toward adenosine A₁ receptor of the intact prodrugs.

Conclusions. We propose 5'-esters of CPA, characterized by suitable lipophilicity and elevated degree of stability in physiological fluids, as possible candidates for CPA prodrugs.

KEY WORDS: N⁶-cyclopentyladenosine; adenosine A₁ receptor; prodrugs; stability; affinity; activity.

INTRODUCTION

Adenosine exerts its physiological effects by interaction with membrane receptors, which have been classified into four subtypes (1). A₁ activation has been found to produce cardiac and neuronal excitability depression (2), and the ability of the prototype A₁-selective agonist 5'-esters of N⁶-cyclopentyladenosine (CPA) to inhibit ischemia has been de-

scribed (3). These effects allow adenosine A₁ agonists to produce ischemic tolerance and protection in neuronal and cardiac tissues (4,5); moreover, selective A₁ agonists have been reported to increase survival of gerbils after ischemic injury (6).

Despite encouraging laboratory results on the central nervous system, the side effects at other organ sites do not allow the clinical use of receptor-subtype-selective adenosine agonists (2,7). Selective A₁ agonists appear poorly adsorbed into the brain (8) and can be quickly degraded *in vivo* or in whole blood: CPA is degraded in rat and human whole blood with an half-life of approximately 20 minutes (9) and in conscious rats with an half-life of 7 minutes (10).

The synthesis of esters is a general approach for developing alcohol prodrugs. This derivatization can provide an increase of stability (11) and/or lipophilicity of the parent compound, thus allowing latency and possibly improving the diffusion through plasma membrane or lipid barriers. An approach aimed to study esters at the 5'-hydroxyl position has been reported (12) and in this context, 5'-acetyl-CPA showed very weak stability at physiological pH values.

We have recently demonstrated that susceptibility to esterases of nucleoside esters prodrugs is strongly dependent on the bulk and lipophilicity of the ester substituent (13). We have therefore investigated a prodrug approach for CPA with the aim to enhance its stability in physiological fluids and thus bioavailability.

MATERIALS AND METHODS

Materials

[³H]CHA (32.3 Ci/mmol) was obtained from NEN Research Products (Boston, MA, USA). [³H]c-AMP (24 Ci/mmol) was obtained from Amersham, Amity Srl (Milan, Italy). CPA, N⁶-cyclohexyladenosine (CHA), and 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) were purchased from RBI (Natick, MA, USA). ADA, c-AMP, Ro 20-1724, and forskolin were obtained from Sigma (St. Louis, MO, USA). HPLC grade solvents were purchased from Carlo Erba Reagenti (Milan, Italy). CHO cells transfected with adenosine A₁ human receptors (CHO A₁) were a kind gift of Prof. Peter Schofield (Garvan Institute of Medical Research, Darlinghurst, Australia) (14,15).

Chemistry

Melting points were determined with a Kofler apparatus and are uncorrected. Ultraviolet spectra were recorded on a Kontron UVIKON 922 spectrometer. Reaction courses were routinely monitored by thin-layer chromatography (TLC) on silica gel precoated Durasil-25 UV₂₅₄ Macherey-Nagel plates with detection under 254 nm UV lamp and/or by spraying the plates with 10% H₂SO₄/MeOH and heating. Nuclear magnetic resonance (¹H-NMR) spectra were determined in DMSO-*d*₆ or CDCl₃ solution with a Bruker AC-200 spectrometer and chemical shifts are given in ppm from internal tetramethylsilane as a standard. Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) spectra were obtained on a Hewlett-Packard HPG2025A mass spectrometer operating in a positive linear mode. Column chromatography was performed with Macherey-Nagel 70-230 mesh silica gel.

¹ Department of Pharmaceutical Sciences, via Fossato di Mortara 17-19, Ferrara University, I-44100 Ferrara, Italy.

² Department of Biology, General Physiology Section, via Borsari 46, Ferrara University, I-44100 Ferrara, Italy.

³ To whom correspondence should be addressed. (e-mail: stefano.manfredini@dns.unife.it)

ABBREVIATIONS: ADA, adenosine deaminase; c-AMP, 3',5'-cyclic adenosine monophosphate; CHA, N⁶-cyclohexyladenosine; CPA, N⁶-cyclopentyladenosine; CNS, central nervous system; DPCPX, 1,3-dipropyl-8-cyclopentylxanthine; 5'-CH-CPA, 5'-cyclohexanoyl-CPA; 5'-PE-CPA, 5'-pentanoyl-CPA; 5'-Oc-CPA, 5'-octanoyl-CPA; Ro 20-1724, 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone; TMOB-CPA, trimethoxybenzoyl-CPA.

Preparation of N⁶-Cyclopentyl-5'-O-Acyl-Adenosine Derivatives (**4a-d**)

Procedure A (**3c-d**)

Compound **2** (**12**) (400 mg, 1 mmol) and 4-DMAP (360 mg, 3 mmol) were dissolved in 15 ml of dry CH₂Cl₂ and the appropriate chloride (2.3 mmol) was added drop-wise under vigorous stirring and the reaction mixture was stirred at room temperature for 3 hours.

Procedure B (**3a**)

In the case of the valeroyl derivative the starting compound was dissolved in 15 ml of anhydrous DMF and valeric anhydride (269 μ l, 1.34 mmol) was added drop-wise under vigorous stirring, the reaction mixture was stirred at room temperature for 20 hours.

When the reaction was complete (TLC: diethyl ether/hexane 8:2), the mixture was diluted with CH₂Cl₂ and then washed with water (3 $\times$ 100 ml); the organic phase, dried over MgSO₄, was evaporated to give 650–800 mg of a crude green oil (**3a-d**). Compounds **3a-d** were used without any further purification for the next deprotection step, the crude compounds were dissolved in a CHCl₃/MeOH solution (24 ml, 1:1 mixture), and 4% TFA/H₂O solution was added (320 ml, 32 ml of TFA solution every 0.1 theoretic mmol of expected product) at 0°C. The reaction was stirred at 4°C for 72 hours (TLC: AcOEt/hexane 8:2). The solvent was then evaporated, below 30°C, and the residue was purified by flash chromatography on silica gel (EtOAc/hexane linear gradient from 6:4 to 8:2). After concentration of the desired fractions the desired compounds were obtained in 40–60% overall yield.

Compound **4a**

Pale yellow foam; 253 mg, 60% yield; UV (MeOH) $\lambda_{\max}$ 269 nm (ϵ : 9000), $\lambda_{\min}$ 232 nm (ϵ : 1900), $\lambda_{\max}$ 212 nm (ϵ : 10100); MALDI MS (M + H)⁺421 Da.

Compound **4b**

Pale yellow foam; 180 mg, 40% yield; UV (MeOH) $\lambda_{\max}$ 269 nm (ϵ : 6500), $\lambda_{\min}$ 240 nm (ϵ : 2500), $\lambda_{\max}$ 213 nm (ϵ : 11200); MALDI MS (M + H)⁺463.7 Da.

Compound **4c**

White foam, 250 mg, 56% yield; UV (MeOH) $\lambda_{\max}$ 269 nm (ϵ : 14800), $\lambda_{\min}$ 232 nm (ϵ : 2100), $\lambda_{\max}$ 214 nm (ϵ : 13900); MALDI MS (M + H)⁺446.9, (M + Na)⁺ 468.9 Da.

Compound **4d**

White solid, mp: 88–93°C, 270 mg, 53% yield; UV (MeOH) $\lambda_{\max}$ 267 nm (ϵ : 14500), $\lambda_{\min}$ 235 nm (ϵ : 4400), $\lambda_{\max}$ 217 nm (ϵ : 19600); MALDI MS (M + H)⁺531.1 Da. The NMR data for compound **4a** are given as an example below

Compound **4a**

¹H NMR (DMSO-*d*₆) δ 8.29 (s, 1H, H8); 8.19 (s, 1H, H2); 7.74 (d, J = 7.83 Hz 1H, NH); 5.9 (d, J = 4.63 Hz, 1H, H1'); 5.58 (d, J = 5.68 Hz, 1H, OH2); 5.37 (d, J = 5.6 Hz, 1H, OH3); 4.72–4.65 (m, 1H, CHNH); 4.45–4.05 (m, 5H, H2', H5', H3', H5'', H4'); 2.26 (t, J = 7.15 Hz 2H, CH₂CO); 1.95–1.91, 1.70–1.40 (m, 8H, cyclopentyl); 1.27–1.16 (m, 4H, 2xCH₂); 0.81 (t, 3H, CH₃, J = 7.09 Hz).

Kinetic Experiments in Human Plasma and Whole Blood

The compounds were incubated at 37°C, 3 ml of plasma or whole blood were spiked with drug solutions resulting in final concentrations of 2 μ M. At regular time intervals 100 μ l of samples were withdrawn. Plasma or whole blood samples were quenched in 200 μ l of ice-cold ethanol or 500 μ l of ice-cold water, respectively, and stored at –20°C until analysis.

CPA and Prodrugs Kinetic Analysis

Degradation of all the compounds was assessed using reversed-phase high performance liquid chromatography (RP-HPLC) separation. As for plasma samples, 50 μ l of 4 $\cdot$ 10^{–5} M internal standard (CHA for CPA and 5'-PE-CPA; 5'-PE-CPA for 5'-Oc-CPA, 5'-CH-CPA and 5'-TMOB-CPA) were added. After 5 minutes of centrifugation at 9,000 g, 250 μ l of solution were reduced to dryness under a nitrogen stream. Two hundred microliters of mobile phase were added and, after centrifugation, 40 μ l were injected into the HPLC system. As for whole blood samples, 50 μ l of 4 $\cdot$ 10^{–5} M internal standard and 50 μ l of 3M sodium hydroxide for CPA analysis or 50 μ l of 10% sulfosalicylic acid for analysis of prodrugs, were added. The samples were extracted twice with 1 ml of water-saturated ethyl acetate. After centrifugation, the organic layer was reduced to dryness and after 100 μ l mobile phase addition, 40 μ l were injected into the HPLC system. This was from Jasco and consisted of a piston pump and a variable wavelength UV detector set at 269 nm. Chromatography was performed at room temperature on a reversed-phase column (Hypersil BDS C-18 5U cartridge column, 150 mm $\times$ 4.6 mm I.D.; Alltech Italia Srl BV, Milan, Italy). The mobile phase consisted of a ternary mixture of acetonitrile, methanol and 10 mM acetate buffer (pH 4) with a ratio of 4/40/56 (v/v/v) for CPA analysis and of 4/50/46 for analysis of prodrugs. The flow rate was 1.2 ml/min. The retention times of CPA, CHA, 5'-Pe-CPA, 5'-Oc-CPA, 5'-CH-CPA and 5'-TMOB-CPA were 4, 7, 5, 20, 9, and 4 minutes, respectively.

Log P Determination for CPA and Prodrugs

Partition coefficients were determined by using MIXXOR separator cylinders (Genex, Industrial Drive, Gaithersburg, MD, USA). After 40 strokes, the residual amounts of the compounds in the aqueous phase were monitored by injection (40 μ l) in the same HPLC system previously described. For CPA and **4c** an octanol/water 1/1 ratio (5 ml each one) was used. For compounds **4a** and **4d** an octanol/water 1/3 ratio (2.5 and 7.5 ml) was used, whereas for compound **4b** an octanol/water 1/9 ratio (1 and 9 ml) was used.

Receptor Binding Assays

Cells were grown and membrane prepared as previously described (16). Membrane aliquots containing 40 μ g of proteins were incubated in 400 μ l of 50 mM TRIS-HCl at 25°C for 105 minutes. Saturation experiments were carried out using twelve different concentrations of [³H] CHA ranging from 1 to 100 nM. Displacement experiments on rat brain membranes were performed in the presence of 20 nM [³H]CHA. Non-specific binding was measured using 10 μ M DPCPX. Separation of bound from free radioligand was per-

formed as previously described (16). The stability of the prodrugs in binding conditions have been evaluated incubating for 105 minutes each compound (10 μ M final concentration) in 400 μ l of TRIS-HCl, pH 7.4, at 25°C in the presence of one membrane aliquot containing 40 μ g of proteins. Forty microliters of filtered solution where injected into the HPLC system.

Determination of Cyclic AMP Levels

Cells grown on 12-well plates were preincubated for 10 minutes at 37°C in fresh serum-free F12/DMEM containing 1 IU/ml of ADA and 10 μ M Ro 20-1724 as phosphodiesterase inhibitor. Then, eight different concentrations of CPA or prodrugs were added. After 5 minutes, forskolin was included (1 μ M final concentration, 500 μ l final volume). The reaction was stopped after 5 minutes by rapid removal of the medium and the addition of 0.3 ml of ice-cold 0.1 N HCl to each well; cells were then scraped, collected, and frozen at -80°C until cAMP assay. The samples were centrifuged for 10 min at 2,000 g, and the supernatants were neutralized with 0.06 ml of 0.5 M Trizma base and assayed for cAMP content, as reported by Brown *et al.* (17).

The stability of the prodrugs in c-AMP measurement conditions have been evaluated incubating each compound (10 μ M final concentration) in 500 μ l of serum-free F12/DMEM on 12-well plates for 10 minutes in the presence of confluent cells. 40 μ l of filtered solution where injected into the HPLC system.

Calculations

Kinetics

The peak areas in the chromatograms were quantified using a chromatographic data processor (Chromatopac C-R3A, Shimadzu, Kyoto, Japan). The half-life of each adenosine analogue was calculated from a semi-logarithmic plot of the peak area ratio between the compound and internal standard, expressed as percentage, *versus* incubation time.

Log P

Peak areas were quantified using the integrator which was calibrated with standard solutions of pure compounds. Log P values have been calculated as

$$\text{Log } P = \frac{(n + C_o) - C_w}{C_w}$$

where C_o and C_w indicate the drug concentration before and after partitioning, respectively $n = 1$ for CPA (**1**) and **4c**, $n = 2.5$ for compounds **4a,d** and $n = 10$ for compound **4b** (18).

Binding and c-AMP Assays

Data of binding experiments were obtained as previously described (16). IC_{50} values in the c-AMP assay were obtained by computer analysis of the concentration-inhibition curves (Graph Pad Prism, San Diego, CA, USA).

RESULTS

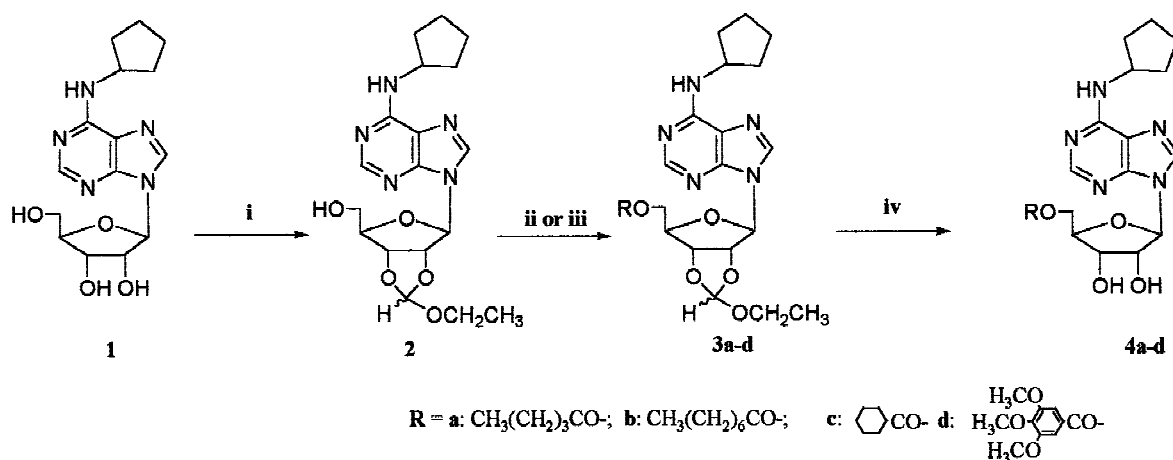
Chemistry

According to a published synthetic strategy (12), CPA (**1**) was reacted with triethylorthoformate in dioxane to give the protected N⁶-cyclopentyl-adenosine (**2**) in 94% yield (Fig. 1). Next treatment of (**2**) with the appropriate acyl-chloride in CH_2Cl_2 and DMAP (19) gave compounds **3b-d**, whereas the 5' pentanoyl ester **3a** was better obtained using the corresponding anhydride in presence of a catalytic amount of 4-DMAP (12). The removal of the protecting group, was performed on the crude ester derivatives, under mild conditions, by using a 1:1 mixture of $CHCl_3$ /MeOH and 4% TFA to give the final products **4a-d** in good yields (two steps 40–60%).

Biology

Table I reports the half life ($t_{1/2}$) values of CPA (**1**) and its prodrugs, obtained by regression analysis of semilogarithmic plots reported in Figure 2. No significant CPA degradation were observed in plasma, whereas $t_{1/2}$ is about 15 minutes in whole blood, where adenosine kinase catalyzes the phosphorylation of the 5'-hydroxyl group of CPA (9).

As for prodrugs, the hydrolysis rate of 5'-esters appeared to be notably modulated by the substituents, the $t_{1/2}$ values



i: Cl_3CCOOH , dioxane, triethylorthoformate; ii: Valeroylic anhydride, DMF dry, 4-DMAP, iii: $RCOCl$, CH_2Cl_2 dry, 4-DMAP, iv: $CHCl_3$ /MeOH 1:1, TFA 4%, 4°C.

Fig. 1. Synthesis of compounds **4a-d**.

Table I Kinetic ($t_{1/2}$), Hydrophobic (Log P), Affinity (K_i), and Activity (IC_{50}) data of CPA and Prodrugs^a

Ligand	$t_{1/2}$ plasma (min)	$t_{1/2}$ whole blood (min)	Log P	K_i (nM)	Percent hydrolysis ^b	IC_{50} (nM)	Percent hydrolysis ^c
1	n.s.	14 ± 3	1.21	2.9 ± 0.2	—	1.5 ± 0.1	n.s.
4a	18 ± 3	0	2.79	586 ± 41	1.14 ± 0.06	70 ± 4	2.59 ± 0.09
4b	28 ± 4	30 ± 4	3.91	176 ± 14	2.30 ± 0.09	76 ± 5	3.97 ± 0.16
4c	45 ± 5	31 ± 4	2.95	941 ± 72	0.33 ± 0.01	82 ± 6	1.56 ± 0.08
4d	n.s.	n.s.	2.27	4260 ± 230	n.s.	2590 ± 120	n.s.

^a Stability data of compounds under experimental condition for affinity and c-AMP levels measurements are also reported. n.s. = not significant within 3 hours.

^b Experimental binding condition.

^c Experimental condition for c-AMP levels determination.

obtained for 5'-Pentanoyl-CPA (**4a**) in plasma (18 minutes) dramatically decreased in whole blood: after 1 minute, the HPLC analysis of the blood sample showed complete disappearance of the prodrug with the concomitant appearance of CPA, that followed, upon 45 minutes, the characteristic degradation pattern. The $t_{1/2}$ values obtained for 5'-Octanoyl-CPA (**4b**) in plasma and whole blood are around 29 min. A similar $t_{1/2}$ value (31 minutes) has been found for 5'-cyclohexanoyl-CPA (**4c**) in whole blood, whereas in plasma this compound showed higher stability ($t_{1/2}$ = 45 minutes). Finally, the presence of the trimethoxyphenyl ring allowed us to obtain a compound with high stability. In fact, degradation of TMOB-CPA (**4d**) was not significant within 3 hours. All prodrugs are more hydrophobic than CPA (LogP = 1.21) with Log P values ranging from 2.27 to 3.91 (Table 1). [³H]CHA was used as A₁ selective radioligand for inhibition experiments, K_D and B_{MAX} values were 24 ± 2 nM and 995 ± 11 fmol/mg protein. Figure 3 illustrates the inhibition experiments of CPA and its prodrug. As reported in Table 1, the K_i value of CPA is 2.9 nM, according to the results obtained from [³H]DPCPX inhibition experiments (16). K_i values of prodrugs, were higher than that of CPA—ranging from 176 to 4260 nM. In order to verify if the K_i values were related on a potential hydrolysis of prodrugs, their stability has been evaluated as the percentage of hydrolysis of the compounds. The weaker compound is not hydrolyzed (5'-TMOB-CPA – **4d** – K_i = 4260 nM) whereas the more potent prodrug showed the higher hydrolysis percentage (5'-Octanoyl-CPA – **4b** – K_i = 176 nM, hydrolysis = 2.3%). The other two pro-

drugs showed intermediate affinity and % hydrolysis values as well (Table 1).

All prodrugs appear able to fully inhibit forskolin-stimulated intracellular c-AMP levels, acting as full agonists; however, their potency is lower with respect to that of CPA (Fig. 4). The IC_{50} value of CPA is 1.5 nM (Table 1), whereas for prodrugs the values ranges from 70 to 2590 nM, the weaker compound is not hydrolyzed (5'-TMOB-CPA, IC_{50} = 2590 nM), and the other three compounds (**4a-c**) show comparable hydrolysis (1.56–3.97%) and thus comparable IC_{50} values (70–82 nM).

DISCUSSION

A series of 5'-esters of CPA have been prepared with the aim to evaluate their chemico-physical properties and stability in physiological fluids. An appropriate synthetic procedure has been adopted in order to avoid concomitant deamination at position N6. Log P values of CPA and its 5'-derivatives have been determined to evaluate the influence of the lipophilicity on the capability to give rise to increased plasma and whole blood concentrations with improved bioavailability as compared to the parent CPA (13). Moreover, affinity and activity were measured and found related to the nature of the substituent at position 5': intact prodrugs, as 5'-TMOB-CPA (**4d**), are not able to interact efficiently with the receptor and thus to display significant activity in cells. Regarding hydrolyzed prodrugs, susceptibility to hydrolysis appeared to be influenced by the chain lengths, indeed the 5'-octanoyl-CPA

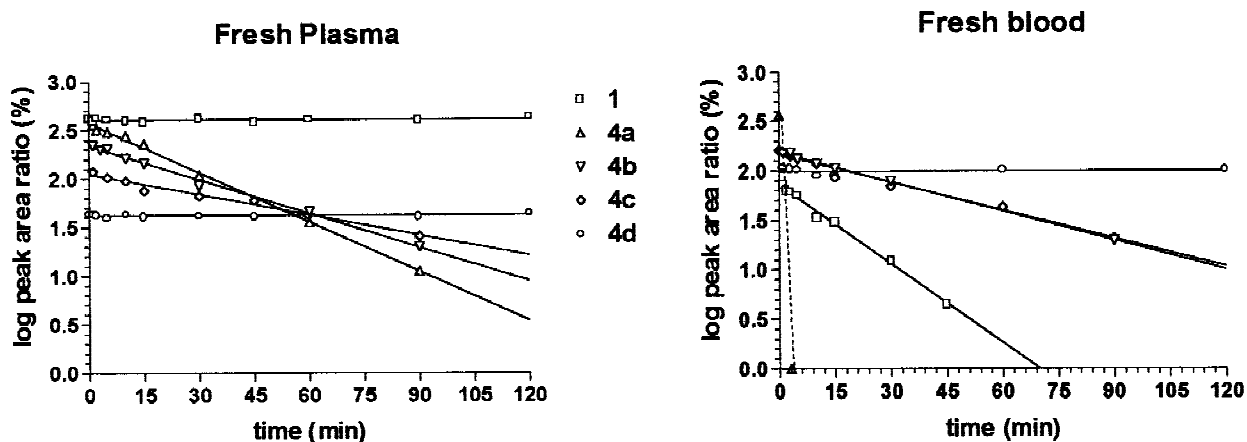


Fig. 2. Time courses of CPA and its prodrugs in human plasma and whole blood.

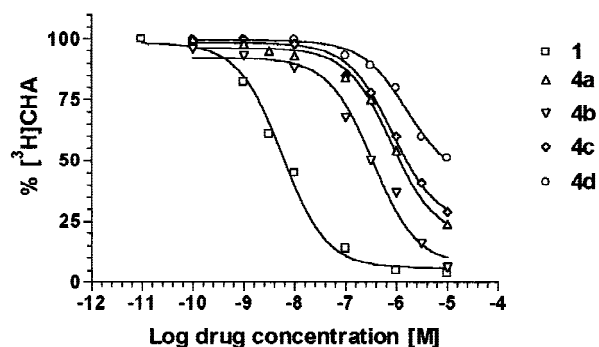


Fig. 3. Competition experiments of CPA and its prodrugs for specific [³H]CHA binding performed at 25°C on CHO A₁ cell membranes.

(4b) was the better hydrolysed, in both experimental conditions for affinity and activity determination, and thus showed the best activity and affinity values among prodrugs. We hypothesise that the activity displayed derive from the different amount of CPA released by the parent prodrug.

Another important result of this study is represented by the influence of the substituent at 5' position on the hydrolysis in plasma and whole blood. 5'-Pentanoyl-CPA (4a) is very rapidly hydrolysed in whole blood (Table 1) whereas is somewhat preserved in plasma ($t_{1/2}$ = 18 minutes). Increasing the chain length, plasma half-life (i.e., 5'-octanoyl-CPA, 4b) is significantly increased ($t_{1/2}$ = 30 minutes) and, surprisingly, a comparable stability in whole blood is also induced. On the other hand, substituents with similar length and LogP, but having a hindered structure (compare 5'-pentanoyl-, 4a, and 5'-cyclohexanoyl-CPA, 4c), induced increased plasma and whole blood half-lives. However, similar stability in whole blood and increased half life in plasma was observed with respect to longer chains such as for the 5'-octanoyl-CPA (4b). These results suggest that chain length strongly influences erythrocyte activity (compare 5'-pentanoyl-, 4a, and 5'-octanoyl-CPA, 4b) whereas steric hindrance contributes to an increasing of plasma half-life. Of particular interest the observation that the octanoyl chain blocked erythrocyte hydrolysis as indicated by the coincident $t_{1/2}$ values in plasma and whole blood. This occurrence was not observed in the case of the 5'-cyclohexanoyl-CPA (4c). It is thus not surprising that a bulky and planar substituent as the TMOB-group induced a great stability.

In conclusion, our results demonstrate that the substitution pattern at 5'-position can highly influence the stability in physiological fluids of CPA derivatives. In our opinion, the octanoyl ester chain is of particular significance because it shows a selective inhibition of CPA release into erythrocytes. This occurrence may be ascribed to a selective process connected either to uptake or hydrolysis inside erythrocytes.

Upon substitution at 5'-position, CPA shows increased lipophilicity, stability and a strong decrease of affinity and activity. CPA properties are restored only after hydrolysis. Taking all these results into account, we therefore propose 5'-esters of CPA, characterized by suitable lipophilicity and high degree of stability in physiological fluids, as candidates for CPA-prodrugs.

ACKNOWLEDGMENTS

This research was supported by Italian Ministry of University, Scientific and Technological Research and by the

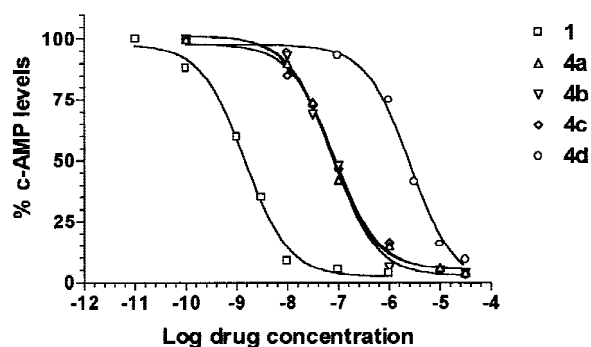


Fig. 4. Inhibition of forskolin-stimulated c-AMP levels in CHO A₁ cells by CPA and prodrugs.

University of Ferrara. The authors thank Prof. Peter Schofield of the Garvan Institute of Medical research, Sydney, Australia and Dr. Andrea Townsend-Nicholson of the Department of Anatomy & Developmental Biology, University College, London, for providing the CHO A₁ cells. The authors thank the Laboratorio Analisi Estense di Ferrara, Italy, for supplying fresh blood.

REFERENCES

1. B. B. Fredholm, M. P. Abbracchio, G. Burnstock, J. W. Daly, T. K. Harden, K. A. Jacobson, P. Leff, and M. Williams. Nomenclature and classification of purinoceptors. *Pharmacol. Rev.* **46**:143–156 (1994).
2. K. A. Jacobson, B. K. Trivedi, P. C. Churchill, and M. Williams. Novel therapeutics acting via purinergic receptors. *Biochem. Pharmacol.* **41**:1399–1410 (1991).
3. R. E. Simpson, M. H. O'Regan, L. M. Perkins, and J. W. Phillis. Excitatory transmitter amino acid release from the ischemic rat cerebral cortex: Effects of adenosine receptor agonists and antagonists. *J. Neurochem.* **58**:1683–1690 (1992).
4. C. Heurteaux, I. Lauritzen, C. Widmann, and M. Lazdunski. Essential role of adenosine, adenosine A₁ receptors, and ATP-sensitive K⁺ channels in cerebral ischemic preconditioning. *Proc. Natl. Acad. Sci. USA* **92**:4666–4670 (1995).
5. A. L. Tucker and J. Linde. Cloned receptors and cardiovascular responses to adenosine. *Cardiovasc. Res.* **27**:54–61 (1993).
6. D. K. von Lubitz, R. C. Lin, N. Bischofberger, M. Beenhakker, M. Boyd, R. Lipartowska, and K. A. Jacobson. Protection against ischemic damage by adenosine amine congener, a potent and selective A₁ receptor agonist. *Eur. J. Pharmacol.* **369**:313–317 (1999).
7. D. K. von Lubitz. Adenosine and cerebral ischemia: Therapeutic future or death of a brave concept? *Eur. J. Pharmacol.* **371**:85–102 (1999).
8. M. S. Brodie, K. Lee, B. B. Fredholm, L. Stahle, and T. V. Dunwiddie. Central versus peripheral mediation of responses to adenosine receptor agonists: Evidence against a central mode of action. *Brain Res.* **415**:323–330 (1987).
9. B. Pavan and A. P. IJzerman. Processing of adenosine receptor agonists in rat and human whole blood. *Biochem. Pharmacol.* **56**:1625–1632 (1998).
10. R. A. A. Mathôt, E. A. van Schaick, M. W. E. Langemeijer, W. Soudijn, D. D. Breimer, A. P. IJzerman, and M. Danhof. Pharmacokinetic-pharmacodynamic relationship of the cardiovascular effects of the adenosine A₁ receptor agonist N⁶-cyclopentyl-adenosine (CPA) in the rat. *J. Pharmacol. Exp. Ther.* **268**:616–624 (1994).
11. G. Remaud, X. Zhou, J. Chattopadhyaya, M. Oivanen, H. Lönnberg. The effect of protecting groups of the nucleobase and the sugar moieties on the acidic hydrolysis of the glycosidic bond of 2'-deoxyadenosine: A kinetic and NMR spectroscopic study. *Tetrahedron* **34**:4453–4461 (1978).
12. M. C. Maillard, O. Nokodijevich, K. F. Lanoue, D. Berkich, X. Ji, R. Bartus, and K. A. Jacobson. Adenosine receptor prodrugs:

- Synthesis and biological activity of derivatives of potent A₁-selective agonists, *J. Pharm. Sci.* **83**:46–53 (1994).
13. S. Manfredini, P. G. Baraldi, R. Bazzanini, F. Bortolotti, S. Vertuani, N. Ashida, and H. Machida. Enzymatic synthesis of 2'-O-acyl prodrugs of 1-(β -D-arabinofuranosyl)-5(E)-(2-bromovinyl)uracil and of 2'-O-acyl-araU, -araC and -araA. *Antiviral Chem. & Chemother.* **9**:25–31 (1998).
 14. A. Townsend-Nicholson and J. Shine. Molecular cloning and characterization of a human brain A₁ adenosine receptor cDNA. *Mol. Brain Res.* **16**:365–370 (1992).
 15. A. Townsend-Nicholson and P. R. Schofield. A threonine residue in the seventh transmembrane domain of the human A₁ adenosine receptor mediates specific agonistic binding. *J. Biol. Chem.* **269**:27900–27906 (1994).
 16. A. Dalpiaz, A. Townsend-Nicholson, M. W. Beukers, P. R. Schofield, and A. P. IJzerman. Thermodynamics of full agonist, partial agonist and antagonist binding to wild-type and mutant adenosine A₁ receptors. *Biochem. Pharmacol.* **56**:1437–1445 (1998).
 17. B. L. Brown, R. P. Ekins, and J. M. D. Albano. Saturation assay for cyclic AMP using endogenous binding protein. In P. Greengard, G. Robinson, and R. Paoletti (eds.), *Advances in Cyclic Nucleotide Research*, Raven Press, New York, 1972 pp. 25–40.
 18. J. I. Wells. *Pharmaceutical Preformulation: The Physicochemical Properties of Drug Substances*, Halsted Press- John-Wiley & Sons, New York, 1988.
 19. V. J. Davisson, D. R. Davis, V. M. Dixit, and C. D. Poulter. Synthesis of Nucleotide 5'-Diphosphates from 5'-O-Tosyl Nucleosides, *J. Org. Chem.* **52**:1794–1801 (1987).