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Enhancement of availability of cloricromene at brain level by a lipophilic prodrug

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Abstract

The pharmacokinetics of a lipophilic alkylamino acid (LAA) prodrug of cloricromene (AD6), namely CLOR-C4, was studied in rat plasma and brain. In particular, we observed that the intraperitoneal administration of CLOR-C4 to rats was able to provide a slight but statistically significant higher concentration of the active drug metabolite (cloricromene acid) in the brain compared with the parent drug administered by the same way. The correlation between pharmacokinetic data and calculated partition (LogP) and brain distribution coefficients (LogBB) supported the hypothesis that the amphiphilic nature of the LAA promoiety could be responsible for a better penetration into the brain, more than the simple increase of lipophilicity gained with respect to the parent drug.

Introduction

Cloricromene (8-monochloro-3-β-diethylaminoethyl-4-methyl-7-ethoxy-carbonyl-methoxycoumarin hydrochloride, AD6) is a synthetic coumarin derivative (Figure 1), which possesses antithrombotic and antiplatelet actions, inhibits polymorphonuclear (PMN) neutrophil function and causes vasodilatation (Sturniolo et al 1989, 1991; Squadrito et al 1993; Calapai et al 1995). AD6 reduces the synthesis of the products of both cyclooxygenase and lipooxygenase pathways (Galli et al 1980; Porcellati et al 1990; Cuzzocrea et al 2000). This drug exerts protective effects in several models of circulatory shock, also inhibiting the induction of inducible nitric oxide synthase (iNOS). AD6 also reduces PMN adhesion to the endothelial cells in a dose-dependent manner (Zatta & Bevilacqua 1999).

Following administration, AD6 is extensively hydrolysed in the bloodstream and tissues to its acid form (CLOR, Figure 1). In platelets and leucocytes, AD6 enters as an ester and is then converted into its acid catabolite (Travagli et al 1989).

In a previous paper, a series of lipophilic conjugates of AD6 with α-alkylamino acids were described (Pignatello et al 2002). The α-amino acids with an alkyl side chain are amphipathic compounds combining the structural and physico-chemical features of lipids with those of amino acids. Their conjugates with drugs possess a high degree of membrane-like character, that may facilitate their passage across membranes and biological barriers, including the blood–brain barrier (BBB) (Toth 1994).

Alkylamino acids bearing a long aliphatic side chain (the so-called lipoamino acids) (Toth 1994) can result in a dramatic loss of solubility in experimental and biological media. We therefore prepared amide conjugates of AD6 with short-chain alkylamino acids (4–6 carbon atoms), to increase the lipophilic character of the drug while keeping a residual solubility. In a preliminary investigation, the conjugates mantained the in-vitro and in-vivo pharmacological pattern of the parent drug (Pignatello et al 2002).

In this study, to assess whether the drug conjugation to lipophilic alkylamino acid (LAA) improves the penetration of AD6 into the brain, the butyl amide conjugate CLOR-C4 (Figure 1) was administered systemically to rats and the resulting level of CLOR in the brain was measured in comparison with that achieved after AD6 administration.

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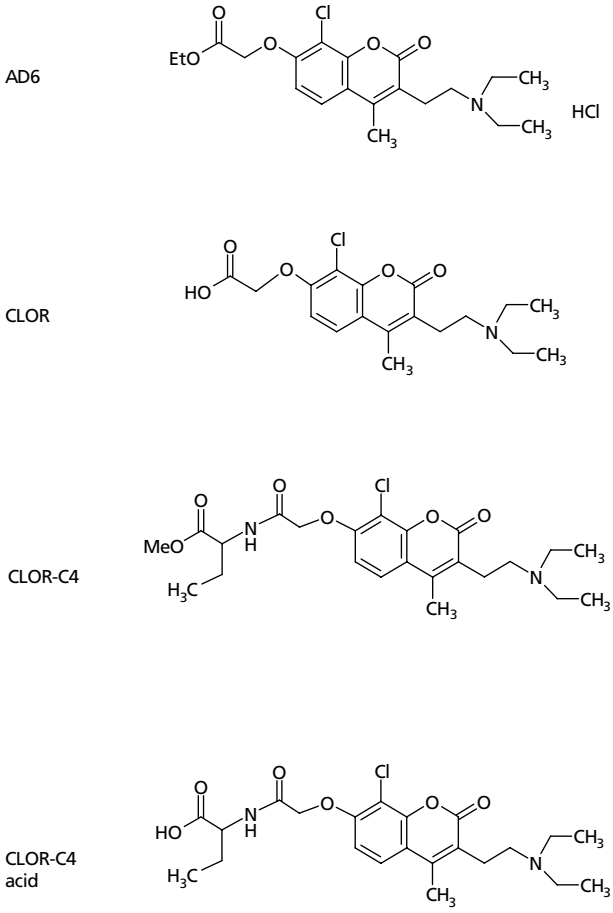

Figure 1 Chemical structure of AD6, CLOR, CLOR-C4 and its acid form.

Materials and Methods

Chemicals

AD6 hydrochloride and cloricromene free acid (CLOR) were gifted by Bausch & Lomb IOM (Catania, Italy). CLOR-C4 was prepared as described previously from the corresponding L-2-aminobutanoic acid methyl ester and CLOR (Pignatello et al 2002). The acid metabolite formed by hydrolysis of CLOR-C4 ester group (CLOR-C4 acid, Figure 1) was obtained by hydrolysis of CLOR-C4 with 1 N NaOH in ethanol at 40°C. The final compound was characterized by IR and ^{1}H NMR analysis.

Calculation of the lipophilicity physico-chemical indicators

Three different software packages were used to validate each other and compensate for the approximation of the databases: ACD LogP 5.15 software (Advanced Chemistry Development Inc., Toronto, Canada); Pallas 3.0 (CompuDrug International, Inc., San Francisco, CA, USA); and OSIRIS Property Explorer (www.actelion.com).

Table 1 Calculated lipophilicity indicators of AD6, its metabolite CLOR, CLOR-C4 and its hydrolysis product CLOR-C4 acid

Compound	cLogP			LogD7.4	cLogBB
	A	B	C	B	D
AD6 (free base)	4.19 ± 0.50	4.66	3.47	2.10	−0.4611 ± 0.0227
CLOR	3.59 ± 0.51	3.61	2.58	−0.38	−1.2704 ± 0.1009
CLOR-C4	4.32 ± 0.72	4.14	2.96	1.89	−0.5857 ± 0.0187
CLOR-C4 acid	3.41 ± 0.77	3.69	2.51	−0.09	−1.0959 ± 0.0823

Values represent the mean ± s.d. Predictions were made using the following software packages: ACD LogP 5.15 (A); Pallas 3.0 (B); Osiris Property Explorer (C); KOWIN 1.57 (D).

The distribution coefficient at pH 7.4 (apparent partition coefficient, $LogD_{7.4}$) was calculated using the Pallas 3.0 software. The partition coefficient between the blood and brain (LogBB) was calculated using the method of Clark (2001) by the KOWIN 1.57 software (Adapted Clark method (F. Hoffmann La Roche Ltd, Basel, Switzerland)) on the test compounds as .smi files. The data are reported in Table 1.

Animals and treatment

Forty adult male Sprague-Dawley rats (Charles River, Calco, Italy), 250–300 g, were used. The rats were treated in accordance with European guidelines for the care and use of laboratory animals (86/609/EEC). The protocol was reviewed by the Institutional Animal Care and Use Committee (IACUC) of the University of Catania and complies with the acceptable standards of animal welfare and human care. CLOR-C4 and AD6 were dissolved in DMSO and 50% aqueous DMSO, respectively, and injected intraperitoneally at a dose of 100 mg kg^{-1} with a single administration. Rats were killed by decapitation at 15, 30 and 60 min after the administration of the compound.

Sample preparation

Brain was excised from rats and stored at −20°C until the analyses. Five hundred milligrams of rat brain, accurately weighed, were added to 1 mL of methanol containing 2% $ZnSO_4$ solution and vortex-mixed vigorously with a pestle until complete homogenization. The homogenate was centrifuged at 10 000 *g* for 5 min and the supernatant was evaporated to dryness under nitrogen. The residue was reconstituted in 300 μL of water–methanol (50:50, v/v), vortex-mixed and centrifuged at 10 000 *g* for 5 min. The supernatant was aspirated with a tuberculine syringe, filtered through a 0.22-μm nylon membrane filter and injected onto the HPLC system.

HPLC assay

The HPLC apparatus was a Hewlett-Packard HP 1100 chromatographic system interfaced to HP Chemstation

software and equipped with a binary pump G1312A, a diode array detector (DAD) G1315A and a thermostatted column compartment G1316A. The HPLC conditions were an adjustment of a previously described method by Maltese & Bucolo (2002). A Hypersil ODS C_{18} reverse-phase column (150 mm × 4.6 mm i.d., 5 μm) and a Hypersil ODS C_{18} column (7.5 mm × 4.6 mm i.d., 5 μm), utilized as a guard column, were purchased from Alltech (Milan, Italy). The mobile phase consisted of 10% CH_3CN and 90% of a water solution containing 1% triethylamine adjusted to pH 3.5 with H_3PO_4; the percentage of CH_3CN was increased to 60% in 10 min at a flow rate of 1 mL min^{-1}. The system returned to 10% in 5 min and was kept under this condition for 3 min to re-equilibrate. The UV detector was set at 318 nm. Chromatography was performed at 25°C. The injected volume was 20 μL.

Calibration curves and recovery

Five hundred milligrams of drug-free rat brain, accurately weighed, were homogenized with 1 mL of methanol containing 2% $ZnSO_4$ and spiked with standard solutions containing CLOR-C4, CLOR or CLOR-C4 acid, in the range of 0.05–4.0 μg mL^{-1}. Spiked brain samples were taken through the assay procedure and calibration graphs were constructed by plotting analyte peak area versus the concentration. Linear regression analysis was used to calculate the slope, intercept and correlation coefficient of the calibration curve. The recovery of analytes from the brain was determined by comparing the peak areas obtained from the direct injection of standard solutions of compounds with those found by extraction from spiked brain. The recovery was approximately 80% for all the analytes.

Stability test

The stability of AD6 and CLOR-C4 in rat brain and plasma was investigated. Spiked samples were prepared with drug-free brain and plasma at two concentrations for each analyte and stored at −20°C. At weeks 0, 1, 2 and 4 the samples were thawed, treated as described in Sample preparation and immediately injected in the HPLC. Both the analytes were stable in rat tissues until analysis.

Statistical analysis

Data are expressed as mean ± s.d. and are measured on an interval scale of measurement. The effect of the pharmacological site (brain or plasma) and drug (AD6 or CLOR-C4) on the pharmacokinetic parameters was examined using a two-way analysis of variance followed by the Student's *t*-test. Statistical significance was accepted at a level of $P < 0.05$.

Results and Discussion

Conjugation of LAA residues to drugs is expected to facilitate the crossing of biological barriers and cell membranes, through an enhancement of passive diffusion. In this respect, positive results have already been obtained using different drugs, such as methotrexate (Pignatello et al 1998, 2000) and peptides (Wong et al 2002; Blanchfield et al 2003; Dulhunty et al 2005).

Table 1 reports the lipophilicity evaluation of the test compounds, expressed as calculated LogP. Although expected differences were given by the three used software packages, a clear trend was observed among the compounds. The conjugation of the cloricromene acid form with 2-aminobutanoic acid slightly increased the lipophilicity (Table 1). Since the acid metabolite, CLOR, has itself a relatively high lipophilic character, what we anticipated from drug conjugation with the alkylamino acid is not a further, strong increase of lipophilicity, but a better balance of its physico-chemical properties, in particular lipophilicity vs hydrophilicity. The partitioning into and binding of a drug to cell membranes/barriers follow complex mechanisms and are related to the so-called anisotropic lipophilicity (Plemper van Balen et al 2004). The latter results not only from the hydrophobicity of the drug but also from its ability to make polar and ionic bonds with the membranes. The enhanced amphipilic character of the conjugate, with respect to the pre drug, can then favour drug penetration across the BBB.

As already shown for some conjugates of tranylcipromine (Pignatello et al 2006a) and idebenone (Pignatello et al 2006b), linking LAA residues to a drug reinforced the amphiphilicity of the latter, producing a more complex interaction with biomembrane models (e.g. DMPC liposomes) and allowing a more complete contact with both polar and lipophilic domains in the membrane phospholipid bilayers. For these reasons, in Table 1 the calculated LogBB values have also been reported. The LogBB value represents a valid parameter to predict the crossing of the BBB and partition into the CNS by a drug administered systemically. Lipophilicity is known to be an important parameter to describe the brain distribution of potential drugs. However, several independent groups have shown that surface properties, as well as charge descriptors, have additionally large impact on the permeability and distribution of drug molecules. With respect to LogP and according to Clark's method (Clark 2001), in the calculation of LogBB the lipophilic parameter is correlated to the polar surface area (PSA) of the molecule, defined as the contribution of oxygen and nitrogen atoms (as well as the hydrogen bond atoms) to the overall molecule surface area. In agreement with literature data, compounds with cLogBB values greater than +0.3 are expected to cross efficaciously the BBB, whereas compounds with values lower than −1.0, which might be due to the presence of hydrogen bonding atoms, poorly distribute in brain tissues.

As Table 1 shows, the calculated LogBB values for CLOR-C4 and AD6 were very close (−0.58 and −0.46, respectively) and both could predict a good penetration in the brain tissues (Trapani et al 2003). Interestingly, while CLOR and CLOR-C4 had cLogP values not particularly different, and in the same order of magnitude as AD6, they strongly differed for the cLogBB value (Table 1) and

the prediction for the ability of CLOR-C4 acid to cross the BBB was less favourable.

An analogous consideration can be made for CLOR-C4 and its corresponding acid form. Both had similar cLogP values (Table 1) but very different cLogBB values. In fact, the free carboxyl groups in CLOR and CLOR-C4 acid are expected to be involved in intra- and intermolecular hydrogen bonds that ultimately would reduce their ability to cross the BBB. This consideration can be further supported by the analysis of the apparent partition coefficients at pH 7.4 ($LogD_{7.4}$, Table 1), a value that resembles better the physiological conditions occurring in the in-vivo test. The two compounds that contained a carboxyl group, CLOR and CLOR-C4 acid, at this pH value were extensively dissociated and behaved as polar compounds, with a consequent lower capacity to accumulate in the brain tissues. As Table 1 shows, AD6 and CLOR-C4 instead still showed a lipophilic character at this pH, which was reflected in the calculated LogBB value and the consequent prediction of a good penetration into the CNS.

The in-vivo findings confirmed first of all that the CLOR–LAA conjugate acted as a prodrug, since a rapid formation of the active metabolite CLOR was observed at plasma and brain levels after the systemic administration of CLOR-C4 to rats. This compound displayed a high stability towards the chemical hydrolysis at various pH values (Pignatello et al 2002), thus completing its profile as a potential prodrug of cloricromene. The second observation relates to the levels of active CLOR measured in rat plasma and brain after the administration of either AD6 or CLOR–C4. It is worth noting that in these experiments the concentrations of drug were measured in excised whole brains, without considering the eventual contribution given by the brain vascular compartment. As Table 2 shows, the administration of the prodrug was able to give a small but statistically significant (Student's *t*-test) increase of CLOR concentration in the brain, from 0.64 to 0.89 $\mu g\,mL^{-1}$, associated with a higher AUC. Concomitantly, the administration of CLOR-C4 gave a slightly lower CLOR concentration and AUC in plasma, compared with the values measured after the administration of the parent drug AD6. The T_{max} values remained unchanged with both drugs (15 min), implying that the rate of formation of CLOR from AD6 or CLOR-C4 was similar.

Table 2 Pharmacokinetic data relative to CLOR measured in rat plasma and brain following the intraperitoneal administration of either AD6 or CLOR-C4

	AD6	CLOR-C4
Plasma		
$AUC_{(0-60)}$ ($\mu g\,min\,mL^{-1}$)	377.34 ± 24.11	365.32 ± 33.61
C_{max} ($\mu g\,mL^{-1}$)	44.34 ± 6.72	41.90 ± 7.32
T_{max} (min)	15	15
Brain		
$AUC_{(0-60)}$ ($\mu g\,min\,g^{-1}$)	6.02 ± 0.20*	7.65 ± 0.41
C_{max} ($\mu g\,g^{-1}$)	0.64 ± 0.20*	0.89 ± 0.30
T_{max} (min)	15	15

Values represent the mean ± s.d., n = 6–8. *$P < 0.01$ vs CLOR-C4.

These findings suggest that the lipophilic prodrug CLOR-C4 was more stable to the hydrolysis in the bloodstream than AD6. The bioconversion pattern of CLOR-C4 is easy to foresee: in fact, after a quick hydrolysis of its ester group to form CLOR-C4 acid, the break of the amide bond to release CLOR is likely to occur at a slower rate because of the lower concentration of amidases in the blood. In the meantime, the more favourable physicochemical properties of the prodrug allowed a higher distribution in brain tissues, where the active metabolite CLOR was finally released.

By comparing these in-vivo results with the calculated physico-chemical properties of CLOR and CLOR-C4 (Table 1), a better correlation was obtained using the cLogBB values than the simple cLogP ones. In other words, although CLOR and CLOR-C4 had similar partition coefficients, the prodrug showed a reduced cLogBB value compared with CLOR. The latter prediction can be well associated with higher levels of the active CLOR form in brain achieved after the administration of the prodrug.

Such findings are in agreement with the initial hypothesis that in linking a drug to LAA promoieties some amphiphilic properties can be acquired, thus reinforcing its interaction with and penetration through biological membranes and barriers.

References

Blanchfield, J. T., Dutton, J. L., Hogg, R. C., Gallagher, O. P., Craik, D. J., Jones, A., Adams, D. J., Lewis, R. J., Alewood, P. F., Toth, I. (2003) Synthesis, structure elucidation, in vitro biological activity, toxicity, and Caco-2 cell permeability of lipophilic analogues of alpha-conotoxin MII. *J. Med. Chem.* **46**: 1266–1272

Calapai, G., Squadrito, F., Rizzo, A., Marciano, M. C., Campo, G. M., Caputi, A. P. (1995) Multiple actions of the coumarine derivative cloricromene and its protective effects on ischemic brain injury. *Naunyn. Schmiedebergs Arch. Pharmacol.* **351**: 209–215

Clark, D. E. (2001) Prediction of intestinal absorption and blood-brain barrier penetration by computational methods. *Comb. Chem. High Throughput Screen.* **4**: 477–496

Cuzzocrea, S., Mazzon, E., Bevilaqua, C., Costantino, G., Britti, D., Mazzullo, G., De Sarro, A., Caputi, A. P. (2000) Cloricromene, a coumarine derivative, protects against collagen-induced arthritis in Lewis rats. *Br. J. Pharmacol.* **131**: 1399–1407

Dulhunty, A. F., Cengia, L., Young, J., Pace, S. M., Harvey, P. J., Lamb, G. D., Chan, Y. N., Wimmer, N., Toth, I., Casarotto, M. G. (2005) Functional implications of modifying RyR-activating peptides for membrane permeability. *Br. J. Pharmacol.* **144**: 743–754

Galli, C., Agradi, E., Petroni, A., Socini, A. (1980) Effects of 8-monochloro-3-beta-diethylamino-ethyl-4-methyl-7-ethoxy-carbonyl-methoxy coumarin (AD_6) on aggregation, arachidonic acid metabolism and thromboxane B_2 formation in human platelets. *Pharmacol. Res. Commun.* **12**: 329–337

Maltese, A., Bucolo, C. (2002) Simultaneous determination of cloricromene and its active metabolite in rabbit aqueous humor by high-performance liquid chromatography. *J. Chromatogr. B* **767**: 153–158

Pignatello, R., Jansen, G., Kathmann, I., Puglisi, G., Toth, I. (1998) Lipoamino acid conjugates of methotrexate with antitumor activity. *J. Pharm. Sci.* **87**: 367–371

Pignatello, R., Spampinato, G., Sorrenti, V., Di Giacomo, C., Vicari, L., McGuire, J. J., Russell, C. A., Puglisi, G., Toth, I. (2000) Lipophilic methotrexate conjugates with antitumor activity. *Eur. J. Pharm. Sci.* **10**: 237–245

Pignatello, R., Puleo, A., Giustolisi, S., Cuzzocrea, S., Dugo, L., Caputi, A. P., Puglisi, G. (2002) Cloricromene conjugates with short-chain alkylamino acids: synthesis and biological evaluation. *Drug Dev. Res.* **57**: 115–121

Pignatello, R., Guccione, S., Castelli, F., Sarpietro, M. G., Giurato, L., Lombardo, M., Puglisi, G., Toth, I. (2006a) Enhancement of drug affinity for cell membranes by conjugation with lipoamino acids. II. Experimental and computational evidences on biomembrane models. *Int. J. Pharm.* **310**: 53–63

Pignatello, R., Intravaia, V. D., Puglisi, G. (2006b) A calorimetric evaluation of the interaction of amphiphilic prodrugs of idebenone with a biomembrane model. *J. Colloid Interface Sci.* In press

Plemper van Balen, G., Marca Martinet, C. A., Caron, G., Bouchard, G., Reist, M., Carrupt, P.-A., Fruttero, R., Gasco, A., Testa, B. (2004) Liposome/water lipophilicity: methods, information content, and pharmaceutical application. *Med. Res. Rev.* **24**: 299–324

Porcellati, S., Costantini, V., Prosdocimi, M., Stasi, M., Pistolesi, R., Nenci, G. G., Goracci, G. (1990) The coumarin derivative AD_6 inhibits the release of arachidonic acid by interfering with phospholipase A_2 activity in human platelets stimulated with trombin. *Agents Actions* **29**: 364–373

Squadrito, F., Altavilla, D., Zingarelli, B., Ioculano, M., Calapai, G., Campo, G. M., Miceli, A., Prosdocimi, M., Caputi, A. P. (1993) The effect of cloricromene, a coumarine derivative, on leukocyte accumulation, myocardial necrosis and TNF-alpha production in myocardial ischaemia-reperfusion injury. *Life Sci.* **53**: 341–355

Sturniolo, R., Squadrito, F., Altavilla, D., Trimarchi, G. R., Prosdocimi, M., Caputi, A. P. (1989) Cloricromene improves survival rate and peritoneal macrophage function in splanchnic artery occlusion shock in rats. *Circ. Shock* **28**: 267–277

Sturniolo, R., Squadrito, F., Campo, G. M., Vinci, R., Calabroni, A., Prosdocimi, M., Caputi, A. P. (1991) Protective effect of cloricromene, a coumarine derivative, in hypovolemic hemorrhagic shock in the rat. *J. Cardiovasc. Pharmacol.* **17**: 261–266

Toth, I. (1994) A novel chemical approach to drug delivery: lipidic amino acid conjugates. *J. Drug Targeting* **2**: 217–239

Trapani, G., Laquintana, V., Latrofa, A., Ma, J., Reed, K., Serra, M., Biggio, G., Liso, G., Gallo, J. M. (2003) Peripheral benzodiazepine receptor ligand-melphalan conjugates for potential selective drug delivery to brain tumors. *Bioconjug. Chem.* **14**: 830–839

Travagli, R. A., Zatta, A., Banzatto, N., Finesso, M., Mariot, R., Tessari, F., Prosdocimi, M. (1989) Molecular aspects of cloricromene (AD6) distribution in human platelets and its pharmacological effects. *Thromb. Res.* **54**: 327–338

Wong, A. K., Ross, B. P., Chan, Y. N., Artursson, P., Lazorova, L., Jones, A., Toth, I. (2002) Determination of transport in the Caco-2 cell assay of compounds varying in lipophilicity using LC-MS: enhanced transport of Leu-enkephalin analogues. *Eur. J. Pharm. Sci.* **16**: 113–118

Zatta, A., Bevilacqua, C. (1999) Differential inhibition of polymorphonuclear leucocyte functions by cloricromene. *Pharmacol. Res.* **40**: 525–533