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Adenosine analogs with covalently attached lipids have enhanced potency at A_1-adenosine receptors

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Chemically functionalized congeners of N^6-phenyladenosine and 1,3-dipropyl-8-phenylxanthine have been covalently coupled to fatty acids, diglycerides, and a phospholipid. The lipid-drug conjugates inhibit *R*-[^{3}H]-phenylisopropyladenosine binding to A_1-adenosine receptors in rat cerebral cortex membranes. A xanthine-phosphatidylethanolamine conjugate bound with a K_i value of 19 nM. Various xanthine esters of low potency are potential prodrugs. Amides of an adenosine amine congener (ADAC) with 18-carbon fatty acids exhibited K_i values at A_1-adenosine receptors of 70 pM, representing a 130-fold enhancement over the affinity of the corresponding acetyl amide. The very high affinity of adenosine-lipid conjugates may be due to stabilization of these adducts in the phospholipid microenvironment of the receptor protein.

Lipid; Adenosine receptor; Xanthine; Adenosine derivative; Lipid-drug conjugate; Prodrug

1. INTRODUCTION

Adenosine receptors are present on the external surface of cell membranes in a variety of organs, including heart, brain, kidneys, lungs and vasculature [1]. Biochemical and physiological studies have defined the presence of two adenosine receptor subtypes. The A_1- (inhibitory) and A_2- (stimulatory) receptor subtypes are linked to adenylate cyclase through guanine nucleotide regulatory proteins [2]. Potent N^6-alkyl and N^6-aryl substituted adenosine agonist analogs are of interest as potential therapeutic agents (e.g. antihypertensive, anxiolytic, or antipsychotic agents). Despite the synthesis of highly potent [3–5] and, to some extent, selective agonists and antagonists, the development of new therapeutic agents has been limited by a lack of organ selectivity.

We report here an approach to increasing affinity of adenosine receptor drugs through the synthesis of covalent lipid conjugates. Due to radically increased hydrophobicity, such lipid conjugates may have unique routes of biodistribution leading to tissue selectivity, as has been seen with other lipid-drug conjugates. In previous studies, conjugation of lipids with antineoplastic drugs [6], L-dopa [7], GABA [8], and other drugs has led to prodrug schemes for intestinal uptake into the lymphatic system or for crossing the blood-brain barrier. For example, a parenterally administered 'lymphotropic' 1,3-distearoyl-glyceride derivative

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Abbreviations: ADAC, N^6-[4-[([4-[([(2-aminoethyl) amio]carbonyl)methyl]anilino]carbonyl)methyl]phenyl] adenosine; R-PIA, *R*-N^6-(1-phenyl-2-propyl)adenosine; XAC, 8-[2-aminoethyl(amino[carbonylmethyloxyphenyl])]-1,3-dipropylxanthine; XCC, 8-[4-(carboxymethyloxyphenyl)]-1,3-dipropylxanthine

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of L-dopa [7] was shown to partition selectively into the brain relative to plasma and produce a favorable therapeutic level in vivo in rodents. It is necessary for L-dopa to be cleaved from this prodrug derivative by enzymatic esterolysis at the site of action. In another example, phospholipid conjugates of immunomodulating muramyl peptides [14] have been used to target these drugs to macrophages in vivo.

The functionalized congener approach to adenosine agonists [4,9] and antagonists [3,10,11] has created distal reactive sites on the drug molecules for generalized covalent attachment. We have synthesized lipid derivatives of such agonists/antagonists by this approach that, a priori, do not require cleavage at the site of action in order to be biologically active. Certain conjugates display affinity at A_1-adenosine receptors far surpassing that of most N^6-substituted adenosines [9].

2. MATERIALS AND METHODS

Lipid conjugates (table 1) of ADAC, compound 4 [9], and XAC, compound 24 [10], were synthesized either through acylation of the primary amino groups on the drug using fatty acid anhydrides or by carbodiimide condensation. The lipid derivatives were purified to chromatographic homogeneity, as judged using thin layer chromatography (silica, chloroform/methanol/acetic acid, 85:10:5), either by crystallization (compounds 6–15, 26–28) or by preparative TLC (compounds 3, 17, 18, 21–23) using glass backed plates, 20 × 20 cm, 1000 μm thick. IR, UV and 300 MHz NMR spectra (measured on a Varian spectrophotometer by W. White and Dr H. Yeh, NIH) were consistent with the assigned structures. Lipid derivatives were stable to storage at −10°C in the solid state for several months.

2.1. *Lipid amides of ADAC* (compounds 6–15)

ADAC (40 mg, 0.07 mmol [9]) was suspended in 5 ml dimethylformamide and treated with a solution of the appropriate fatty acid anhydride (0.16 mmol) in 2 ml of methylene dichloride. The mixture was warmed to 40°C until the reaction was judged to be complete by thin layer chromatography (typically 10 min). The product was isolated as a precipitate upon addition of methylene dichloride and ether.

2.2. *1,3-Dimyristin-2-XCC* (compound 17)

1,3-Dimyristin (45.5 mg, 89 μmol) and XCC ([10], 34 mg, 89 μmol) were added to 3 ml dimethylformamide containing 20%, v/v, chloroform (ethanol-free). Excess EDAC (50 mg) and 4-dimethylaminopyridine (20 mg) were added, and the mixture was stirred for 24 h. Additional chloroform (3 ml) was added and the mixture was extracted three times with phosphate buffer (pH 7). The organic layer was evaporated and the residue was recrystallized from chloroform/methanol and washed with water. The crude yield was 42 mg (49%). A homogeneous sample was obtained by preparative thin layer chromatography (silica, $CHCl_3$/MeOH/HOAc, 85:10:5).

2.3. *1,2-Distearoyl-3-XCC-phosphatidylethanolamine* (compound 18)

XCC [10] was coupled to 1,2-distearoyl phosphatidylethanolamine (Calbiochem) using the EDAC method (see above, compound 17), and the product was purified by preparative TLC.

2.4. *Elaidic ester of 8-[2-hydroxyethyl(amino-[carbonylmethyloxyphenyl])]-1,3-dipropylxanthine* (compound 22)

8-[2-Hydroxyethyl(amino[carbonylmethyloxyphenyl])]-1,3-dipropylxanthine ([11], 74 mg, 0.17 mmol) and elaidic anhydride (215 mg, 0.39 mmol, Sigma, St Louis, MO) were added to an equivolume mixture of methylene dichloride and dimethylformamide (10 ml) and treated with 4-dimethylaminopyridine (30 mg). After stirring for 1 day the reaction was shown by thin layer chromatography to have reached approx. 70% completion. Water was added, and the organic phase was washed successively with sodium bicarbonate and sodium bisulfate. The organic layer was reduced in volume, and the product was purified by preparative thin layer chromatography ($CHCl_3$/MeOH/HOAc, 90:5:5). A high R_f band was isolated and eluted with methanol/chloroform (1:1). The solvent was evaporated, and the residue was collected and washed with a minimal amount of cold methanol. The NMR spectrum of the product (60 mg, 50% yield) was consistent with the

assigned structure. The IR spectra showed the expected ester carbonyl resonance band.

2.5. *Xanthine lipid amides* (compounds 26–28)

The compounds were prepared by acylation of XAC using fatty acid anhydrides (see above) or by the following procedure for myristoyl-XAC, compound 26: XAC ([10], 191 mg, 0.45 mmol), myristic acid (123 mg, 0.54 mmol) and 1-hydroxybenzotriazole (60 mg, 1 eq.) were suspended in 20 ml dimethylformamide. EDAC (0.18 g, 2 eq.) was added and the mixture was stirred for 24 h. Saturated sodium bicarbonate solution (40 ml) was added, the solids were collected by filtration, washed (H_2O), and dried in vacuo at 50°C. The product (0.28 g, 98% yield), melting at 228–230°C, was homogeneous by thin layer chromatography (R_f = 0.75, silica, $CHCl_3$/MeOH/HOAc, 85:10:5). Analysis ($C_{35}H_{54}N_6O_5 \cdot 3/2H_2O$) calc. 63.13% C, 8.63% H, 12.62% N; found 63.19% C, 8.47% H, 12.30% N.

Competitive binding assays on rat brain membranes were carried out as reported [3,11] using 1 nM *R*-[^{3}H]phenylisopropyladenosine (PIA) as the radioligand. The membrane homogenate was incubated in the presence of the test compound for 90 min at 37°C in 50 mM Tris-HCl buffer at pH 7.4. The membranes were filtered through Whatman GF/B filters. IC_{50} values were determined in no less than three determinations, each done in triplicate, and converted to K_i values using a K_d value for [^{3}H]PIA of 1.0 nM and the Cheng-Prusoff equation. Effects on cyclic AMP accumulation in adipocyte and PC12 (pheochromocytoma) cell membranes were measured as described [12].

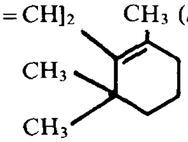
3. RESULTS AND DISCUSSION

Synthetic yields and results of the binding assays (Partitioning of lipid-drug conjugates into the membranes (not in association with receptor) and/or intracellular internalization and/or formation of micelles in solution would affect binding equilibrium, thus K_i values are to be taken as apparent values.) are shown in table 1. *p*-(Carboxymethyl)phenyladenosine [9] was coupled to distearoyl phosphatidylethanolamine to give the conjugate 3. This conjugate, used also for attempted receptor targeting of liposomes [14], was considerably less potent than the parent N^6-phenyladenosine, 2.

The high potency of the adenosine amine congener (ADAC, 4) has been related to the presence of a distal amino group and two *para*-substituted aromatic rings in the functionalized chain [9]. The amino group is probably involved in an electrostatic interaction with an anionic site, such as a charged amino acid residue or a phospholipid in the membrane. The enhancing effect of a distal amino group on affinity at adenosine receptors has been noted also for xanthine antagonists [3]. Acylation of the terminal primary amino group of ADAC with a series of aliphatic carboxylic acids of various lengths gave amide derivatives 5–15. The potencies of these amides at A_1-adenosine receptors were found to be dependent upon the length of the attached carboxylic acid moiety. Long chain lipid conjugates, such as the 18-carbon monoolefinic elaidoyl derivative, 10 (fig.1), were extremely potent at displacing [^{3}H]PIA from brain membranes in comparison to simpler *N*-acyl derivatives or to N^6-cyclohexyladenosine, 1. Hill coefficients for compounds 10–13 were in the range of 0.9 to 1.0. Affinity for the A_1 binding site was enhanced 130-fold upon extension of the fatty acid chain from acetyl-ADAC, 5, 11-fold less potent than ADAC, to stearoyl-ADAC. Lipids of 18 carbon atoms (compounds 9–13) differing only in the number (between 0 and 3) and geometry of olefinic groups present displayed comparable potencies. Thus, enhancement of potency caused by conjugation with a lipid appears not to be affected greatly by steric factors on the receptor microenvironment. As shown in fig.1, potency of ADAC amides at A_1-receptors increased gradually between lengths of eight (capryloyl-ADAC, compound 6) and twelve carbons (lauroyl-ADAC, compound 8).

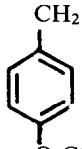
The amide of ADAC with a branched-chain, cyclic fatty acid (retinoic acid), 15, displayed very high potency. The longest amide synthesized, behenoyl-ADAC, 14, and to a much lesser extent, stearoyl-ADAC, 9, had unusual inhibition curves (fig.2). Compound 14 displaced [^{3}H]PIA binding at subnanomolar concentrations, but at increased concentrations (between 1 nM and 1 μM) the inhibition of radiotracer binding at A_1 sites was diminished (reaching a minimum at 30 nM). A plausible explanation for this unusual behavior is

Table 1
Structures[a] and binding affinities of purine-lipid conjugates

Compounds	% yield	K_i (nM)
Adenosine derivatives		
1. N^6-cyclohexyl (CHA)	–	1.2
2. N^6-phenyl	–	3.2 ± 0.5
3. N^6-C_6H_4-CH_2CONH-$(CH_2)_2OPO_3CH_2CHOCO(CH_2)_{16}CH_3$ with $CH_2OCO(CH_2)_{16}CH_3$ on the CH	43	84 ± 4
4. N^6-C_6H_4-CH_2CO-C_6H_4-$NH(CH_2)_2NH$-H (ADAC)	–	0.85 ± 0.35
5. N^6-C_6H_4-CH_2CO-C_6H_4-$NH(CH_2)_2NH$-$COCH_3$ (ADAC-$COCH_3$)	–	9.3 ± 1.7
6. ADAC-$CO(CH_2)_6CH_3$	83	5.8 ± 1.2
7. ADAC-$CO(CH_2)_8CH_3$	22	0.79 ± 0.06
8. ADAC-$CO(CH_2)_{10}CH_3$	95	0.217 ± 0.027
9. ADAC-$CO(CH_2)_{16}CH_3$	90	0.071 ± 0.004
10. ADAC-$CO(CH_2)_7CH=CH(CH_2)_7CH_3$ (*trans*)	100	0.069 ± 0.008
11. ADAC-$CO(CH_2)_7CH=CH(CH_2)_7CH_3$	83	0.079 ± 0.005
12. ADAC-$CO(CH_2)_7CH=CHCH_2CH=CH(CH_2)_4CH_3$	59	0.082 ± 0.005
13. ADAC-$CO(CH_2)_7CH=CHCH_2CH=CHCH_2CH=CHCH_2CH_3$	89	0.070 ± 0.005
14. ADAC-$CO(CH_2)_{20}CH_3$	51	0.39 ± 0.03[b]
15. ADAC-CO[-CH=C(CH_3)-CH=CH]$_2$ CH_3 (*trans*) 	30	0.13 ± 0.02
Xanthine derivatives (1,3-dipropyl)		
16. 8-C_6H_4-OCH_2CO-OCH_2CH_3 (XCC-OEt)	–	42 ± 3
17. 8-C_6H_4-OCH_2CO-$OCH[$-$CH_2OCO(CH_2)_{12}CH_3]_2$	71	104 ± 7.7
18. 8-C_6H_4-OCH_2CO-$OCH_2CHOCO(CH_2)_{16}CH_3$ with $CH_2OCO(CH_2)_{16}CH_3$ on the CH	21	910 ± 40
19. 8-C_6H_4-OCH_2CO-$NH(CH_2)_2OH$	–	10.2 ± 0.8
20. 8-C_6H_4-OCH_2CO-$NH(CH_2)_2OCOCH_3$	63	16.3 ± 1.3
21. 8-C_6H_4-OCH_2CO-$NH(CH_2)_2OCO(CH_2)_{16}CH_3$	32	820 ± 140
22. 8-C_6H_4-OCH_2CO-$NH(CH_2)_2OCO(CH_2)_7CH=CH(CH_2)_7CH_3$ (*trans*)	70	121 ± 13

(*continued*)

Table 1 (continuation)

23. 8-  -$OCH_2CO\text{-}NH(CH_2)_2OPO_3CH_2CHOCO(CH_2)_{16}CH_3$ $CH_2OCO(CH_2)_{16}CH_3$	27	19	± 2
24. 8-  -$OCH_2CO\text{-}NH(CH_2)_2NH\text{-}H$ (XAC)	–	1.2	± 0.5
25. 8-  -$OCH_2CO\text{-}NH(CH_2)_2NH\text{-}COCH_3$ (XAC-$COCH_3$)	–	24	± 3.5
26. XAC-$CO(CH_2)_{12}CH_3$	98	98	± 16
27. XAC-$CO(CH_2)_7CH=CHCH_2CH=CH(CH_2)_4CH_3$	30	24	± 2.3
28. XAC-$COCH_2NHCOCHNH\text{-}CO(CH_2)_7CH=CHCH_2CH=CH(CH_2)_4CH_3$ CH_2  O-$CO(CH_2)_7CH=CHCH_2CH=CH(CH_2)_4CH_3$	40	179	± 18

[a] All olefin bonds are *cis* unless noted
[b] Complex curve, see text

that the longer straight-chain aliphatic conjugates may coalesce into micelles at critical concentrations as low as 1 nM, thus effectively removing ligand from solution.

Compounds 6, 9 and 10 at 100 nM were found to inhibit isoproterenol-stimulated adenylate cyclase through A_1-adenosine receptors in adipocyte membranes. Thus, these compounds

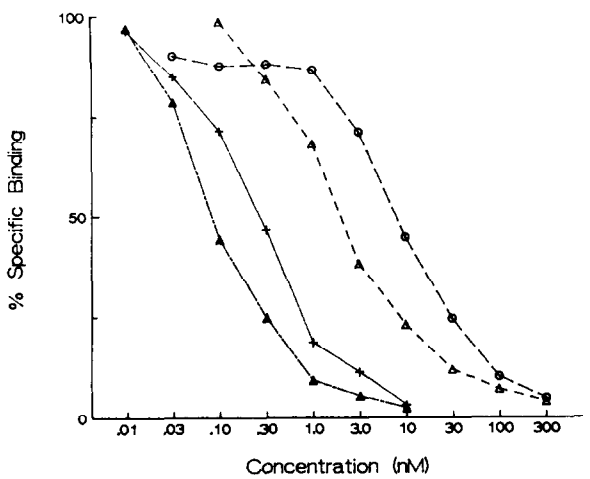


Fig.1. Effect of chain length of the fatty acid coupled to ADAC in the displacement of [^{3}H]PIA from rat cerebral cortex membranes. No. of carbon atoms in lipid: 8, capryloyl-ADAC, compound 6 (○); 10, caproyl-ADAC, compound 7 (△); 12, lauroyl-ADAC, compound 8 (+); 18, mono-olefin, elaidoyl-ADAC, compound 10 (▲).

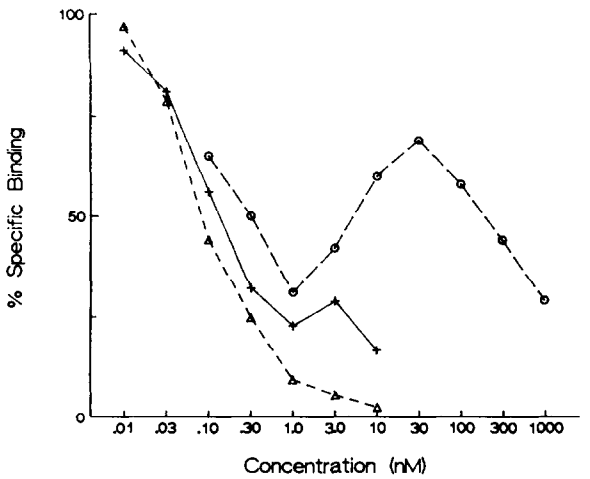


Fig.2. Secondary rise in inhibition curves for long chain, saturated fatty acids coupled to ADAC in the displacement of [^{3}H]PIA from rat cerebral cortex membranes. ADAC amide derivative: elaidoyl, compound 10, C-18, unsaturated (△); stearoyl, compound 9, C-18, saturated (+); behenoyl, compound 14, C-22, saturated (○).

have agonist activity at A_1-adenosine receptors. In A_2-mediated stimulatory effects on adenylate cyclase in PC12 cells, compounds 9 and 10 were agonists with less than full efficacy (EC_{50} values of 1.0 and 1.6 μM, respectively). The adenosine derivatives 3 and 6, acting as partial agonists, and the xanthine derivative 23, acting as an antagonist, reversed the stimulatory effect of 5'-*N*-ethylcarboxamidoadenosine with K_B values of 380 + 30, 570 + 50, and 295 + 30 nM, respectively.

With xanthine-lipid conjugates, derived from XCC (compounds 17, 18 and 21–23) and XAC (compounds 25–28), the degree of receptor affinity observed for adenosine-lipid conjugates was not achieved. Among the xanthine conjugates are lipid derivatives of diverse structures, including glycerol esters (17, 18), fatty acid amides (26–28), esters (17, 18, 20–23), and a phospholipid derivative (23). A complex fatty acid bis-adduct (compound 28) of a XAC-dipeptide (L-Tyr-Gly) is included. In this series, the xanthine-lipid conjugates of highest affinity at A_1 receptors are the XCC amide of phosphatidylethanolamine, 23, and myristoyl-XAC, 27. The xanthine ester derivative, such as compound 21 of which the esterolysis product, 19, is considerably less potent, are designed as prodrugs.

An explanation for the high potency of the fatty acid amide derivatives of ADAC may lie in the interaction of the pendant lipid with the phospholipid membrane in which the receptor protein lies. Thus, the long hydrophobic group could act as a distal anchor in the membrane microenvironment of the receptor. Such high potency in binding was not paralleled with xanthine derivatives. This may reflect a different orientation of the attached chain at the receptor binding site or a conformational difference between agonist and antagonist-occupied receptors.

A catalytic mechanism of binding involving hydrophobic association of various amphiphilic peptide hormones with lipid membranes has been deduced [15]. We have now shown that the potency of an agent (adenosine) that acts at a discrete receptor protein at the cell surface may be enhanced by covalent attachment to lipids. The potential use of these high affinity lipid derivatives for selective organ delivery currently is under investigation.

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