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Solubilities of Adenosine Antagonists Determined by Radioreceptor Assay

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Abstract—The practical use of many adenosine receptor antagonists is limited by poor aqueous solubility. In some cases, solubilities are so low that they are difficult to measure by conventional means. To determine solubilities of adenosine antagonists, a sensitive radioreceptor method has been developed. Solubilities in Tris buffer (pH 7·7) ranged from 141 nM for 8-(2-amino-4-chlorophenyl)-1,3-dipropylxanthine to 945 μM for the amino-substituted xanthine PD 113,297. Ratios between solubility and adenosine receptor affinity varied from 15·8 for the A_2-selective antagonist HTQZ to 169 000 for PD 113,297. From literature data on functional activity, it is apparent that useful adenosine antagonist activity in-vivo is only seen in compounds with solubility/affinity ratios greater than 100.

Exogenous adenosine has numerous effects in the cardiovascular, gastrointestinal, endocrine, reproductive, and nervous systems. Most of these responses can be understood in the context of adenosine's role in maintenance of adequate tissue oxygenation and energy charge (Newby 1984; Sparks & Bardenheuer 1986; Bruns 1987). However, in many cases it is not clear under what conditions sufficient endogenous adenosine is produced to elicit these responses; that is, actions of adenosine could be physiological, pathological, or even irrelevant. In addition, roles for adenosine in pathological conditions could be beneficial or exacerbative.

For this reason, adenosine receptor antagonists are being sought as pharmacological tools to determine the roles of adenosine in-vivo. Although caffeine and theophylline have long been known to block adenosine receptors (Sattin & Rall 1970), their use as tools is questionable due to their weak potency and low specificity (Rall 1982). Because of these problems, it is not clear whether their actions (for instance, CNS stimulation, bronchodilation, and diuresis) are due to adenosine blockade (for a review, see Bruns 1987).

Although more potent adenosine antagonists have been identified (Bruns 1981; Bruns et al 1983, 1986, 1987a, b; Hamilton et al 1985; Jacobson et al 1985, 1986; Martinson et al 1987; Williams et al 1987), in many cases (for example, see Bruns et al 1983) the desired goal of in-vivo activity has not been achieved due to poor physicochemical properties, such as lack of aqueous solubility. In fact, aqueous solubilities of some antagonists are below the detection limits of standard methods such as HPLC with UV detection. In the present study, solubilities of several important adenosine antagonists have been determined by a sensitive method involving precipitation and radioreceptor assay. The results indicate that a favourable ratio between solubility and receptor affinity appears to be a prerequisite for useful adenosine antagonist activity in-vivo.

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Materials and Methods

Materials

CGS 15943 and D-Lys-XAC were generous gifts of Dr Michael Williams, Ciba-Geigy, Summit, NJ, USA, and Dr Kenneth Jacobson, NIDDK, NIH, Bethesda, MD, USA, respectively. Other adenosine antagonists were synthesized at Parke-Davis, except for alloxazine (purchased from Aldrich, Milwaukee, WI, USA), theophylline (Sigma, St. Louis, MO, USA), and XAC (Research Biochemicals, Inc., Natick, MA, USA). Structures of antagonists are illustrated in Fig. 1. [^{3}H]N^6-Cyclohexyladenosine ([^{3}H]CHA) and [^{3}H]-1-(6-amino-9H-purin-9-yl)-1-deoxy-*N*-ethyl-β-D-ribofuranuronamide ([^{3}H]NECA) (both about 40 Ci mmol^{-1}) were from New England Nuclear, and DMSO was from Aldrich (Gold Label grade). Tris buffer was made from Sigma pH 7·7 preset crystals.

Adenosine receptor binding

Affinities of compounds for adenosine A_1 receptors were determined in [^{3}H]CHA binding in rat whole brain membranes (Bruns et al 1986). A_2 affinities were determined using binding of [^{3}H]NECA to rat striatal membranes in the presence of 50 nM N^6-cyclopentyladenosine (Bruns et al 1986).

Solubility determination by HPLC with UV detection

The adenosine antagonist was suspended at about 1 mg mL^{-1} in 50 mM sodium phosphate pH 7·4 and shaken in a glass tube on a rotating wheel for 3 h. The sample was centrifuged and the concentration of antagonist determined by reverse-phase HPLC with UV detection.

Solubility determination by radioreceptor assay

Adenosine antagonists were dissolved in DMSO and diluted 1:100 into 50 mM Tris-HCl pH 7·7 (Tris buffer) in glass scintillation vials to a final concentration about 10 times higher than the anticipated limit of solubility. The vials were sealed with polypropylene cone-lined caps and incubated in a shaking water bath at 25°C. After approximately 18 h, the suspension was removed and centrifuged, and aliquots of the

supernatant were diluted in Tris for testing in [^{3}H]CHA binding. Each antagonist was tested at six concentrations chosen to encompass the IC50. For comparison, six-point concentration-inhibition curves were also carried out for each antagonist that was dissolved and diluted in DMSO. All incubations were adjusted to a 1% final concentration of DMSO. The order of additions was DMSO (10–20 μL), Tris buffer (0–10 μL), supernatant (1000 μL), [^{3}H]CHA (200 μL, 10 nM), and rat brain membranes (780 μL), 20 mg original wet weight (with 0·2 units of adenosine deaminase). For control curves, the order was antagonist (20 μL in DMSO), Tris buffer (1000 μl), [^{3}H]CHA, and membranes. Samples were incubated for 1 h at 25°C and filtered on Whatman GF/B glass-fibre filter sheets on a Brandel 48R cell harvester (Bruns et al 1986). Solubilities (S) were calculated from the concentration (C) at which the precipitation was carried out, the apparent IC50 (A) of the supernatant from the precipitated sample, and the true IC50 (T) of the antagonist obtained by dilution in DMSO (eqn 1).

$$S = C \times T/A \quad (1)$$

An example of this calculation is the following: in one experiment, 8-cyclopentyl-1,3-dipropylxanthine (CPX) was dissolved at 10 mM in DMSO, diluted to a concentration of 100 μM in Tris buffer, and incubated overnight. The apparent IC50 of the supernatant from this sample was 7·56 nM, whereas the true IC50 from a sample that was diluted in DMSO was 0·715 nM. From the ratio 0·715/7·56 it is evident that only 9·41% of the receptor binding activity remained in the supernatant after precipitation. The solubility of CPX in this experiment was therefore 100 μM × (0·715 nM/7·56 nM) = 100 × 0·0941 = 9·41 μM.

Results

Although theophylline is a relatively weak adenosine receptor antagonist (A_1 affinity 8·5 μM; see Table 2), its solubility in distilled water is 46 nM (Windholz et al 1983), resulting in a solubility/A_1 ratio of 5400. This high ratio of solubility to receptor affinity undoubtedly contributes to the good in-vivo activity of theophylline.

When more potent adenosine antagonists were reported, it was immediately apparent that many of these were far less soluble than theophylline. For instance, it was evident from simple visual inspection that 8-phenyltheophylline formed a precipitate in physiological buffer when diluted from DMSO or NaOH to concentrations of 20 μM or more, implying a solubility for this compound of about 10 μM. Because poor solubility was a serious drawback to the use of these antagonists as tools in adenosine research, we wished to study the factors controlling their solubility.

Several considerations led us to investigate new methods for determining solubilities of adenosine antagonists. Determination of solubility by visual inspection is only a semi-quantitative measure, and is unreliable for compounds with solubilities below about 10 μM because the precipitate may be too scant to be visible at these concentrations. The standard method for quantitative determination of solubility involves measurement of the concentration of dissolved compound by HPLC with UV detection (see Methods); however, this method also has sensitivity limits in the low micromolar range. Since at least one compound of interest, 8-(2-amino-4-chlorophenyl)-1,3-dipropylxanthine (PACPX), was expected to have a solubility below 1 μM (Bruns et al 1985), we developed a method for solubility determination based on a sensitive A_1 radioreceptor assay.

Briefly, the method involves precipitation of the antagonist by dilution from DMSO into buffer, followed by centrifugation. The concentration of antagonist in the supernatant is determined in [^{3}H]CHA binding. This method can be used to determine solubility for any compound with a ratio of solubility to A_1 affinity greater than 2, regardless of the absolute solubility of the compound.

Solubilities of adenosine antagonists varied markedly (Table 1). The least soluble antagonist was PACPX, with a solubility of only 141 nM. Several potent adenosine antago-

Table 1. Solubility determinations for adenosine receptor antagonists. Solutions of adenosine antagonists in DMSO were diluted 1 : 100 into Tris buffer at the indicated test concentrations, incubated overnight at 25°C, and centrifuged to remove precipitate. Solubility was calculated by comparison of the apparent IC50 in [^{3}H]CHA binding for the supernatant with a control IC50 for a sample diluted in DMSO. Because of its poor A_1 affinity, the solubility of HTQZ was determined in A_2 binding. All values are geometric means of 3 or more independent experiments, except for those of HTQZ and CGS 14943, which are from single determinations. The coefficient of variance of the mean (CVM) is expressed as percent of the mean; as an illustration, an IC50 of 100 nM with a CVM of 25% indicates that the 67% confidence interval falls between 100/1·25 and 100 × 1·25, or between 80 and 125 nM. Structures of antagonists are given in Fig. 1.

		IC50, nM (CVM)		
Compound	Test conc μM	Supernatant	Control	Solubility, μM (CVM)
PD 113,297	1000	9·52 (31·0%)	9·00 (6·1%)	945 (31·9%)
CPT	1000	41·6 (16·1%)	19·2 (5·4%)	461 (17·1%)
alloxazine	1000	216000 (5·1%)	11300 (9·2%)	52·2 (10·6%)
CPEQ	100	94·2 (15·4%)	47·7 (15·5%)	50·7 (22·5%)
PD 115,199	100	94·3 (14·0%)	26·7 (10·1%)	28·3 (17·6%)
CPX	100	4·29 (42·7%)	0·734 (5·9%)	17·1 (43·4%)
DPX	100	1020 (23·0%)	78·6 (9·7%)	7·73 (25·4%)
8-PT	100	2230 (7·1%)	120 (10·6%)	5·38 (13·0%)
APPP	100	641 (33·0%)	28·3 (26·4%)	4·41 (44·7%)
CPQ	100	369 (20·8%)	14·6 (10·0%)	4·00 (23·6%)
BPA	100	7420 (15·5%)	271 (13·7%)	3·66 (21·3%)
HTQZ	100	10100	198	1·96
CGS 15943	100	398	6·94	1·74
PACPX	10	470 (11·6%)	6·61 (7·9%)	0·141 (14·3%)

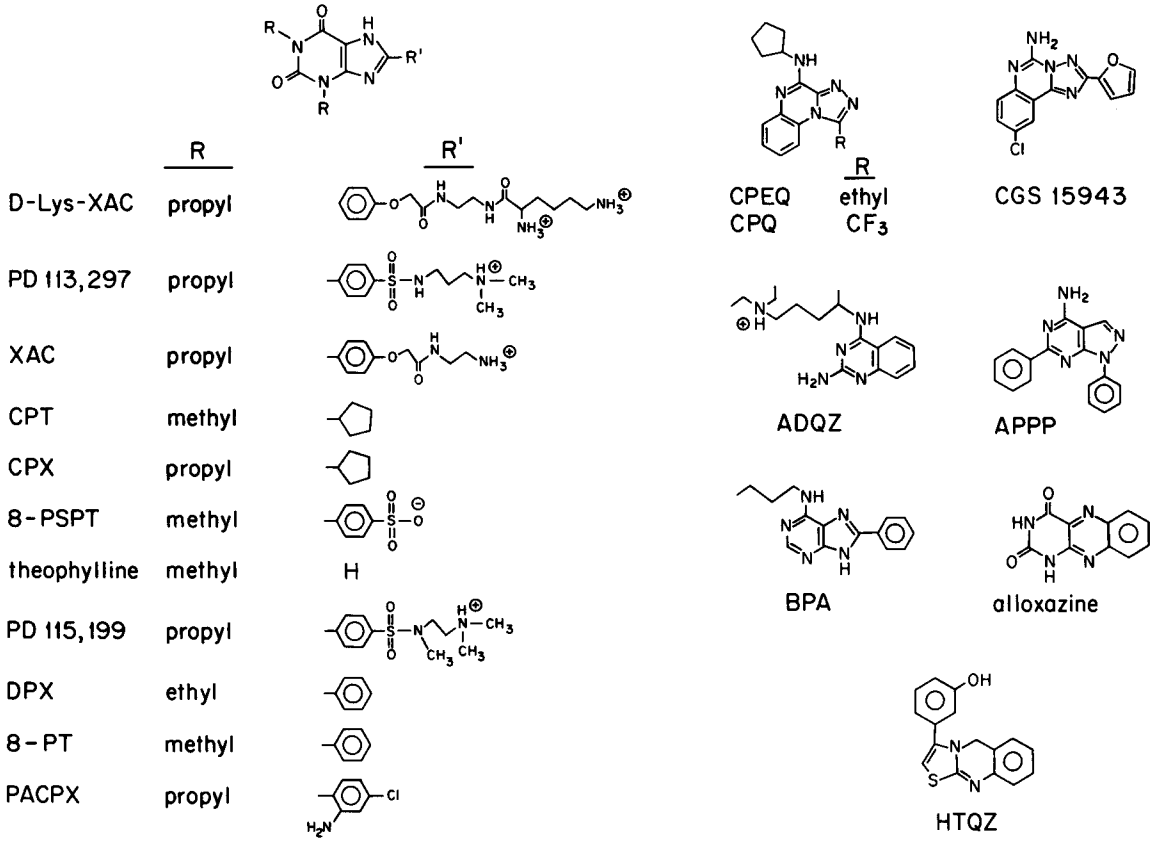

FIG. 1. Structures of adenosine receptor antagonists.

nists, including PD 113,297 (Fig. 1) and 8-cyclopentyltheophylline (CPT), had solubilities near 1 mM, and the charged xanthine 8-(*p*-sulphophenyl)theophylline had a solubility about equal to that of theophylline. The solubilities of 965 μM for PD 113,297 and 461 μM for CPT are in reasonably good agreement with respective values of 630 μM and 403 μM determined by the HPLC/UV method (A. M. Young, personal communication). Several non-xanthine adenosine antagonists (Bruns 1981; Bruns & Coughenour 1987; Bruns & Hamilton 1987; Williams et al 1987; Trivedi & Bruns 1988) had solubilities in the low micromolar range.

Perhaps more important than absolute solubility is the ratio between solubility and adenosine receptor affinity (Table 2). The highest ratio determined in the present study (169 000) was exhibited by PD 113,297, while CPT and CPX also had ratios greater than that of theophylline. The xanthine congener D-Lys-XAC, the solubility of which was determined by conventional means (K. A. Jacobson, personal communication), had an even higher ratio of 360 000 (Table 2). The poorest ratio was that of HTQZ (15·8). Despite the high A_1 affinity of PACPX, its extremely low solubility resulted in a mediocre solubility/A_1 ratio of only 56.

Discussion

In this study, we report the solubilities of several important adenosine receptor antagonists determined by a sensitive radioreceptor method. These results corroborate the idea that solubility is an important determinant of in-vivo activity. In particular, antagonists with solubility/affinity ratios of less than 100 appear to have poor in-vivo activity. For example, 8-phenyltheophylline has a ratio of 63 and has not shown in-vivo antagonist activity except under drastic conditions, i.e., parenteral administration in sodium hydroxide vehicle (Lautt & Legare 1985; Fredholm et al 1987; Collis 1988). To our knowledge, PACPX (ratio 56) has never been shown to block adenosine responses in-vivo. Conversely, CPT and CPX have solubility/A_1 ratios of over 30 000 and have been shown to be potent A_1 antagonists in-vivo (Bruns et al 1988). Other compounds that have been shown to possess adenosine antagonist activity in-vivo include PD 113,297 (T. Mertz, personal communication), XAC (Evoniuk et al 1987b; Fredholm et al 1987), 8-PSPT (Evoniuk et al 1987a), and CGS 15943 (Ghai et al 1987), all of which possess solubility/affinity ratios greater than 1000.

From the data in Table 2, it is clear that the measure that is most pertinent to in-vivo antagonist activity is not solubility alone or affinity alone, but rather the ratio between the two. For instance, PACPX has a higher A_1 affinity than CPT, but only the latter is active in-vivo. CPX is only slightly more soluble than 8-PT, yet is markedly more potent as an antagonist in-vivo.

The solubility/affinity ratio is of course only one of many potential determinants of in-vivo activity. Other relevant variables include partition coefficient and metabolic stability. However, among the adenosine antagonists in the present study, in-vivo activity appears to be limited mainly

Table 2. Ratios of solubility to adenosine receptor affinity for adenosine antagonists. Solubilities are from Table 1, except that the solubilities of 8-PSPT and ADQZ were determined by the HPLC/UV method, the solubility of theophylline was from the Windholz et al (1983), and solubilities of XAC and D-Lys-XAC in 0·1 M sodium phosphate, pH 7·4, were provided by Dr K. A. Jacobson. A_1 and A_2 affinities were determined in [^{3}H]CHA binding and [^{3}H]NECA binding, respectively (Bruns et al 1986). K_i values for D-Lys-XAC, XAC, and CGS 15943 are from single determinations, and other K_i values are triplicate determinations from Bruns et al (1986, 1987a); Bruns & Coughenour (1987); Bruns & Hamilton (1987); Trivedi & Bruns (1988).

		K_i (nM)		Ratio	
Compound	Solubility (μM)	A_1	A_2	solub/A_1	solub/A_2
D-Lys-XAC	520	1·44	86	360000	6000
PD 113,297	945	5·6	70	169000	13500
XAC	90	0·86	27	105000	3300
CPT	461	10·9	1440	42000	320
CPX	17·1	0·46	340	37000	50
8-PSPT	>30000	2600	15300	>10000	>2000
Theophylline	42500	8500	25000	5000	1700
CPEQ	50·7	24	3000	2100	16·9
PD 115,199	28·3	14	16	2000	1770
CGS 15943	1·74	3·9	1·51	450	1150
ADQZ	265	600	310	440	850
CPQ	4·00	7·3	1000	550	4·0
APPP	4·41	23	35	192	126
DPX	7·73	44	860	176	9·0
8-PT	5·38	86	850	63	6·3
PACPX	0·141	2·5	92	56	1·53
BPA	3·66	173	1050	21	3·5
Alloxazine	52·2	5200	2700	10·0	19·3
HTQZ	1·96	3000	124	0·65	15·8

by solubility. For instance, 8-PT and CPT both have partition coefficients (log P) near 2·0, yet only the latter has good activity in-vivo.

Several of the compounds in Table 2 (e.g. D-Lys-XAC and PD 113,297) have been made water soluble by attaching a charged side-chain to the insoluble 1,3-dipropyl-8-phenylxanthine. Although this strategy has resulted in good activity by the i.v. and i.p. routes, it is possible that the charged moiety will impede transport from the intestinal lumen to the circulation and from the circulation to the brain. For instance, XAC and PD 113, 297 do not appear to cross the blood-brain barrier (Fredholm et al 1987; Seale et al 1989; T. G. Heffner, personal communication). This is not a problem with neutral compounds such as theophylline and CPT, which show good oral activity and are active as antagonists in behavioral tests (Bruns et al 1988).

The solubility values in the present paper should be useful in the design of experiments involving adenosine antagonists. We are aware of several published studies in which adenosine antagonists have been used at concentrations above their solubility limits. For instance, Burnstock & Hoyle (1985) observed that concentrations of PACPX above 2 μM did not cause any further rightward shift in the concentration-response curve for adenosine in the guinea-pig atrium. Although these results were interpreted as evidence for a non-competitive mode of action for PACPX, they could also have been due to precipitation of PACPX at the higher concentrations. Precipitation at 4–10 μM would probably not be visible to the unaided eye. In contrast to its actions in guinea-pig atria, PACPX showed concentration-dependent blockade up to 10 μM in guinea pig taenia coli (Burnstock & Hoyle 1985). The latter result suggests that under some circumstances supersaturated concentrations of PACPX might remain dissolved for a significant length of time. Such a delayed precipitation of PACPX has been noted by Hoyle et al (1988).

PACPX was originally synthesized in an attempt to maximize adenosine receptor affinity without regard to solubility (Bruns et al 1983). [A second phase of this effort concentrated on the development of more-soluble antagonists such as CPT, PD 113,297, and XAC (Bruns et al 1985; Hamilton et al 1985; Jacobson et al 1985, 1986).] PACPX exemplifies an important pitfall for the medicinal chemist: maximizing receptor affinity in-vitro without regard to other properties often results in unacceptable physicochemical properties such as poor solubility or exceptionally high partition coefficient. This phenomenon may be due to the fact that receptor binding is a process of partitioning of a ligand between water and the binding site of the receptor. If the receptor binding site is less polar than water, then any increase in hydrophobicity will make a molecule less likely to enter the water phase, thereby tending to increase receptor affinity. However, it will also make the molecule less likely to enter the water phase in a water: solid partition, resulting in insolubility.

The forces that control solubility of xanthines have not been studied extensively. However, xanthines are purines, and some of the forces that stabilize the double helix of DNA may also act to stabilize the xanthines in the crystalline state. In particular, inter-base hydrogen bonding and base stacking appear to make important contributions to insolubility of xanthines. The importance of hydrogen bonding is illustrated by the order of solubility of methylated xanthines: 1,3,7-trimethylxanthine > 1,3-dimethylxanthine > 1-methylxanthine > xanthine (respective solubilities 112 mM, 46·3 mM, > 500 μM, and 453 μM; Windholz et al 1983, and unpublished observation). The increase in solubility with each additional methyl group occurs despite the added hydrophobicity conferred by replacing a hydrogen with a methyl, and is due to the fact that each methyl group eliminates a proton that otherwise could form a hydrogen bond between two molecules in the crystal. The importance

of base stacking is illustrated by the 7000-fold loss of solubility of 8-phenyltheophylline compared with theophylline. The flat phenyl ring of 8-phenyltheophylline presumably extends the region of base stacking beyond the purine ring, thereby stabilizing the crystalline state and drastically reducing solubility. The loss of solubility with 8-phenyl substitution is not due solely to increased hydrophobicity, since 8-cyclopentyltheophylline is about 80-fold more soluble than 8-phenyltheophylline (Table 1). The cyclopentyl group is more hydrophobic than the phenyl group (Hansch & Leo 1979), but presumably stacks less well due to its lesser planarity.

Although the present study concerns only adenosine antagonists, the radioreceptor method described here could be adapted to determine solubilities of other compounds with poor aqueous solubility but with a high affinity for a receptor or other binding site. Examples might include steroids, phorbol esters, ligands for the 2,3,7,8-tetrachlorodibenzo-*p*-dioxin receptor, and other agents relevant to pharmacology and toxicology.

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