Probing the adenosine receptor with adenosine and xanthine biotin conjugates

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Biotin-containing analogs of a potent agonist (N^6-phenyladenosine) and a potent antagonist (1,3-dipropyl-8-phenylxanthine) of adenosine receptor activity have been synthesized. A spacer chain to the biotin moiety is attached in both cases to the *para*-position of the phenyl ring. Two biotin conjugates of N^6-phenyladenosine differing only in the length of the spacer chain bind to the adenosine receptor and to avidin simultaneously. The shorter-chain derivative was more potent in inhibiting binding of N^6-[^{3}H]cyclohexyladenosine to rat cerebral cortical membranes (K_i of 11 nM in the absence of avidin, 36 nM for the avidin complex). Three biotin conjugates of 1,3-dipropyl-8-phenylxanthine bound competitively to the adenosine receptor, but only in the absence of avidin. The results are interpreted in terms of the possible orientation of the ligands at the receptor binding site.

Adenosine receptor *Biotin* *Avidin* *Functionalized congener* N^6-*Phenyladenosine* *Xanthine*

1. INTRODUCTION

The non-covalent avidin-biotin complex [1] forms rapidly, is remarkably stable, and has been used in a variety of manners as biochemical probes [2,3]. Biotin may be attached readily through its carboxyl group to a biologically active compound (pharmacophore) to afford a bifunctional entity, which still retains high affinity for avidin through its biotin component, and which has the potential to retain activity for its target site (receptor, enzyme, transport protein) through its pharmacophore (see [4–11] for examples of use in receptor localization and purification and in drug delivery).

We have introduced previously a series of ligands for the adenosine receptor which feature adenosine or xanthines covalently attached to chains bearing carboxylic or amino groups [12,13]. These biologically active analogs may be attached to other molecules or 'carriers' through the carboxyl or amino groups: such analogs are termed functionalized 'congeners' following the nomenclature of Goodman and co-workers [14]. Functionalized congeners of N^6-phenyladenosine (agonists) and of 1,3-dialkyl-8-phenylxanthine (antagonists) bearing amino groups were chosen as the parent ligands for the attachment to biotin. The properties of biotin conjugates of adenosine receptor ligands in the absence and presence of avidin were used to investigate the accessibility of the ligand binding site(s) on adenosine receptors.

2. MATERIALS AND METHODS

Compounds **2a** and **3a** were prepared as reported previously [12,13] by acylation of the appropriate amino congener by d-biotin *N*-hydroxysuccinimide ester. Compounds **2b** and **3b** were synthesized in an analogous manner from the same functionalized congeners except that d-biotinyl-ϵ-aminocaproic acid *N*-hydroxysuccinimide ester (Calbiochem) was the acylating agent. Compound **3c** was derived from the glycyl-glycine conjugate [12] of the 1,3-dipropylxanthine amino congener and d-biotinyl-ϵ-aminocaproic acid *N*-hydroxysuccinimide ester. A typical acylation reaction con-

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sisted of suspending 10–50 mg of the free amino-functionalized analog in several ml of dimethylformamide and treating the suspension with 1.2 molar equivalents of either active ester derivative of biotin. After the reaction mixture was stirred for one day the product precipitated upon addition of small amounts of methanol and ether. The conjugates were recrystallized from dimethylformamide/ether, and the purity of each product was ascertained by thin layer chromatography (silica gel, chloroform/methanol/acetic acid, 85:10:5) using a biotin-specific spray reagent [15] and ultraviolet light for visualization. The following solid products were obtained: **2b**, 93% yield, mp 195–198°C; **3b**, 99%, mp 218–221°C; **3c**, 85%, mp 210–212.5°C. The biotin conjugates gave correct C, H, and N analyses (**2b**, **3b**, and **3c** as the monohydrates) and were characterized further by 360 MHz proton NMR.

Avidin (affinity purified), streptavidin, avidin-fluorescein isothiocyanate, and avidin-peroxidase (purified by affinity chromatography and gel permeation chromatography) were obtained from Sigma.

Assays of competitive binding of N^6-[^{3}H]cyclohexyladenosine on rat cerebral cortical membranes were carried out as described previously [13] using triplicate determinations. Binding assays in the presence of avidin were carried out after preincubating the biotin conjugate at varying concentrations and avidin at a fixed concentration for 10 min in 50 mM Tris buffer (pH 7.7).

Binding of biotin derivatives to avidin was measured by a modification of the procedure of Rylatt et al. [16] which involves saturation of unoccupied sites with [^{3}H]biotin (New England Nuclear, 35 Ci/mmol) after preincubation of the avidin with varying amounts of the biotin derivative. The stable biotin-avidin complex was precipitated using alkaline zinc sulfate and isolated as a pellet by centrifugation. The pellet was resuspended and centrifuged to reduce baseline counts. Concentrations and conditions were as described by Kohanski and Lane [7].

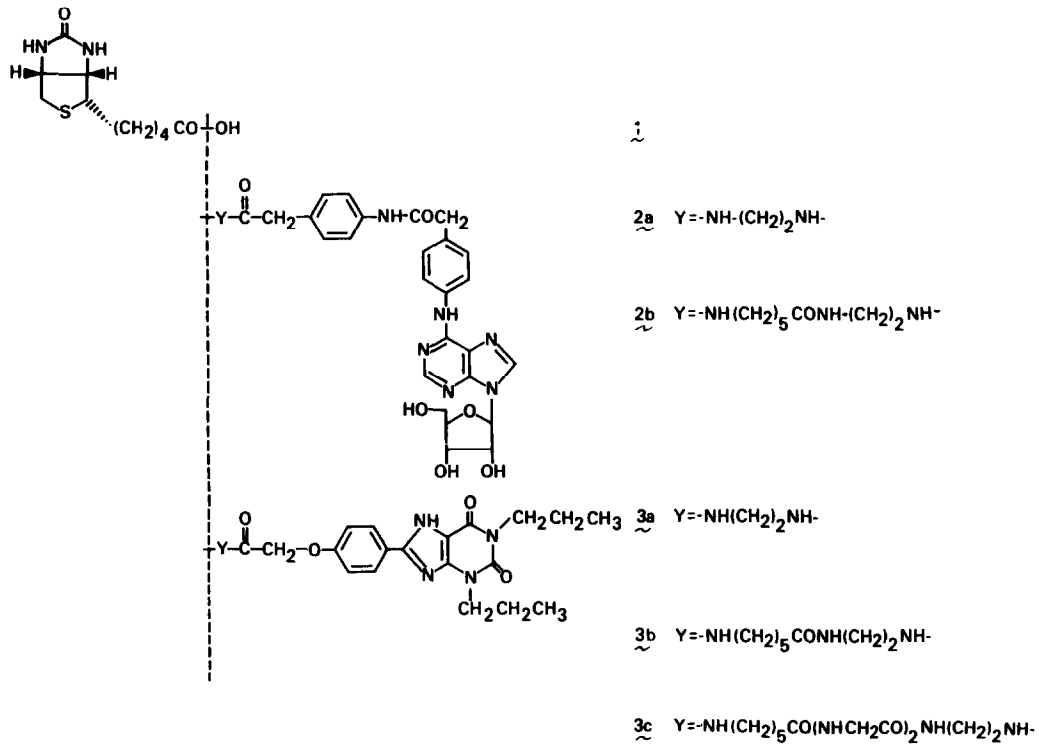

Fig.1. Structures of biotin and biotin conjugates.

3. RESULTS

Biotin was coupled to amino congeners of N^6-phenyladenosine (agonists) and 1,3-dipropyl-8-phenylxanthine (antagonists) to give compounds **2a** and **3a**, respectively. Similarly, conjugates **2b** and **3b** containing the ϵ-aminocaproyl spacer group [6] were prepared to examine the relationship between chain length and activity in the binding assay. In the antagonist series, an analog (**3c**) containing glycylglycine as an additional spacer was also prepared.

The ability of the 5 biotin conjugates to inhibit competitively binding of N^6-[^{3}H]cyclohexyladenosine (CHA) to rat cerebral cortical membranes was used as a measure of the affinity for the central A_1-adenosine receptor ([13] and refs therein). The adenosine conjugates (**2a**, **2b**) had substantial affinity for the A_1-receptor (table 1) albeit slightly reduced relative to the parent N^6-phenyladenosine ($K_i = 3.2 \pm 0.5$ nM). The diminished potency of the conjugate having the aminocaproyl spacer group (**2b**) suggests a positive contribution of the biotin group when closer to the pharmacophore.

The xanthine conjugates (**3a**, **3b**, and **3c**) were several-fold less potent at the A_1-adenosine receptor than the parent 1,3-dipropyl-8-phenylxanthine ($K_i = 13 \pm 3$ nM). Remarkably, the presence of the ϵ-aminocaproyl spacer group in **3b** does not affect the receptor affinity. Compound **3c** having the longest spacer chain was the least potent in the antagonist series in binding at the A_1-adenosine receptor. The xanthine congeners were also active in an A_2-adenosine receptor response, viz., reversal of the cyclic AMP accumulation elicited by 2-chloroadenosine in guinea-pig brain slices [13] (K_i for compound **3a** was 180 ± 80 nM).

Binding of the isolated conjugates to avidin was studied through the zinc sulfate precipitation of the stable complex [16]. Binding sites not occupied by a biotin conjugate molecule were titrated with [^{3}H]biotin (fig.2). All 5 of the biotin conjugates bound to the avidin tetramer in the predicted ratio of 4:1.

To assess the efficiency of binding of the conjugates simultaneously to the adenosine receptor and to avidin, the conjugates were pre-incubated with various concentrations of avidin to preform the complex and then tested in the competitive binding assay with [^{3}H]cyclohexyladenosine. For compound **2a** a family of curves resulted (fig.3), in

Table 1

Affinity constants for biotinylated ligands, from competitive binding assays of N^6-[^{3}H]cyclohexyladenosine to rat cerebral cortical membranes

Compound	K_i (nM)	
	No avidin	1 μM avidin
2a	11.4 ± 0.4	36
2b	18 ± 1.7	35
3a	54 ± 2	> 500
3b	50 ± 0.4	> 500
3c	60	> 500

Values are the mean of single experiments done in triplicate or the mean ± SE of 2-6 experiments

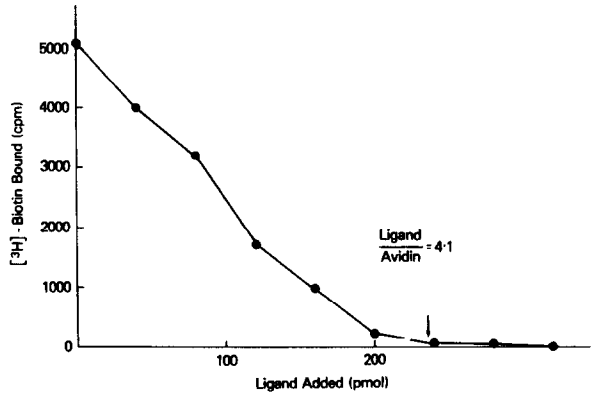

Fig.2. Binding of compound **2a** to avidin (60 pmol).

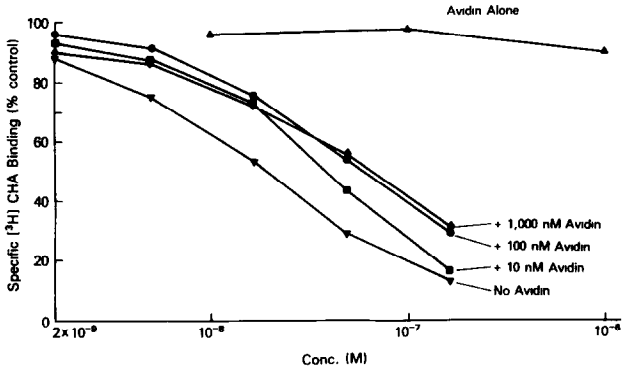

Fig.3. Inhibition of binding of N^6-cyclohexyladenosine to rat cerebral cortical membranes by compound **2a**.

which the binding profile was shifted to the right by avidin. At a large excess of avidin (100 nM in avidin tetramer, each containing 4 independent binding sites) the apparent K_i for **2a** had increased from 11 to 36 nM. The long cahin biotin-adenosine conjugate (**2b**) also displayed simultaneous binding to avidin and to the adenosine receptor (fig.4). Avidin alone had no effect on binding of [^{3}H]cyclohexyladenosine until it reached micromolar concentration at which point 10–20% inhibition occurred.

In contrast, the biotin-xanthine conjugates regardless of the length of the spacer chain bound to the receptor only in the absence of avidin. At a concentration of 1 μM in avidin subunits, the xanthine conjugate **3a** (fig.5) did not cause 50% inhibition even at a concentration 8-fold greater than the IC_{50} in the absence of avidin.

The most potent adenosine conjugate, compound **2a**, was then tested for A_1-receptor binding

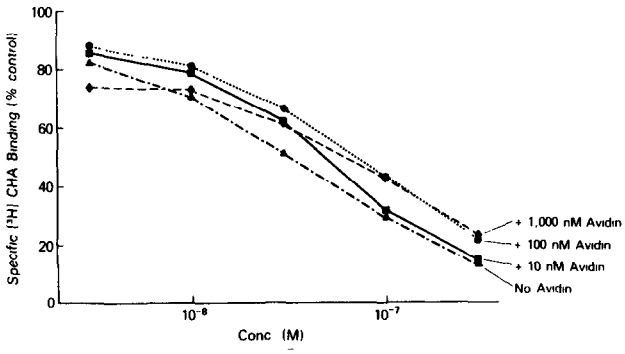

Fig.4. Inhibition of binding of N^6-cyclohexyladenosine to rat cerebral cortical membranes by compound **2b**.

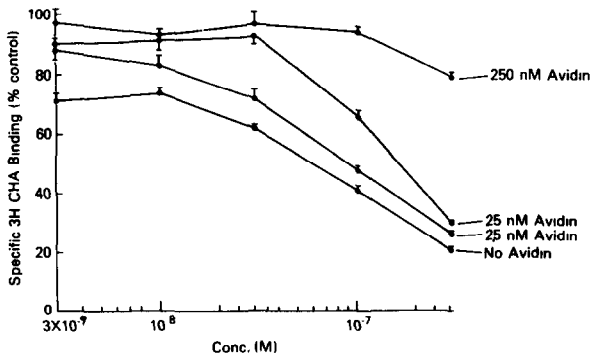

Fig.5. Inhibition of binding of N^6-cyclohexyladenosine to rat cerebral cortical membranes by compound **3a**.

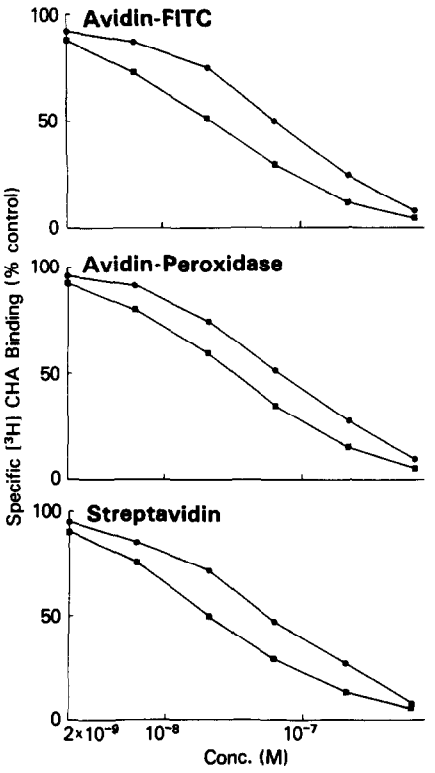

Fig.6. Effects of the presence of avidin derivatives (100 nM) on the inhibition of binding of N^6-cyclohexyladenosine to rat cerebral cortical membranes by compound **2a**. ■, adenosine-biotin conjugate; ●, adenosine-biotin conjugate + avidin derivative; ◆, avidin derivative alone.

in the presence of several avidin derivatives used in histochemical studies. Avidin coupled to fluorescein isothiocyanate (average 3.5 equivalents per tetramer) or to peroxidase (average 1.5 equivalents per tetramer) was preincubated with compound **2a** prior to the binding assays. The tetrameric protein streptavidin from *Streptomyces avidinii*, having less non-specific binding [17] than the glycoprotein avidin, was also examined. Streptavidin, like avidin, is a tetramer with 4 binding sites for biotin. These derivatives of avidin are virtually indistinguishable from avidin in the effect on binding of compound **2a** to the receptor (fig.6).

4. DISCUSSION

Pharmacophores attached to biotin have proven useful for the investigation of the topography of

macromolecular regognition sites [5], for histochemical localization [4] and for the purification of recognition sites [7]. Biotin conjugates of an adenosine receptor agonist and an adenosine receptor antagonist have now been prepared and used to investigate possible topographical constraints at agonist and antagonist binding sites of A_1-adenosine receptors.

The adenosine conjugate **2a** readily binds simultaneously to the A_1-adenosine receptor and to avidin: the K_i of **2a** is reduced only by a factor of 3 in the presence of excess avidin. In the simplest interpretation, the spacer chain of 17 atoms separating the biotin moiety from the N^6-amino group of adenine is sufficiently long to bridge the combined depth of both binding sites. It has been estimated [6] that the carboxyl group of biotin in the bound state is located at a maximum distance of 2 Å from the surface of the avidin molecule. This distance leads to an estimated maximum limit of the depth of the binding site at the adenosine A_1-receptor. From our results, the N^6-amino group of adenosine should be located in the binding site at a depth of no greater than approx. 12 Å, based on the sum of the linear dimensions of the spacer chain in the extended conformation. These dimensions might be defined more narrowly through the synthesis and biochemical characterization of adenosine conjugates with even shorter spacer chains.

The inability of the xanthine conjugates (**3a**, **3b**, and **3c**), having spacer chains up to 24 atoms in length, to bind simultaneously to the A_1-adenosine receptor and to avidin is surprising in view of results with the adenosine conjugates. One explanation is that the agonist and antagonist sites may be at different domains of the receptor, perhaps with the agonist site 'buried' much deeper. Alternatively, the domains may be the same, but the antagonist may bind so that the 8-phenyl moiety is 'buried' more deeply within the site than is the N^6-phenyl moiety of the agonist. Regardless of the interpretation, the present results provide evidence that a bulky macromolecule, i.e., the avidin-biotin complex, some 28 Å from a potent antagonist pharmacophore (**3c**) prevents interaction at the A_1-adenosine receptor. In contrast, a bulky macromolecule (avidin-biotin complex) some 14 Å from a potent agonist pharmacophore does not prevent interaction of the A_1-adenosine receptor. Whether other receptors will show analogous results with biotinylated agonists and antagonists deserves investigation.

The present results also indicate that the ligand binding site of the adenosine receptor is located within of 12 Å the surface of the membrane. Extracellular adenosine receptors were demonstrated previously through the cardiovascular activity of non-penetrating oligosaccharide conjugates of adenosine and theophylline [18].

The accessibility of the adenosine receptor to agonist-biopolymer complexes suggests that considerable flexibility is possible in the design of receptor probes having bulky substituent groups at the end of a chain and in the design of carrier-bound therapeutic agents acting at the adenosine receptor. In particular, the biotinylated conjugates of adenosine have considerable potential as a bifunctional link between the adenosine receptor and avidin coupled to spectroscopic or enzymatic markers for localization studies or biochemical assays.

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