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4-[6-(Dansylamino)hexylamino]-7-methyl-2-phenyl-1,8-naphthyridine as a new potential fluorescent probe for studying A_1-adenosine receptor

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Abstract

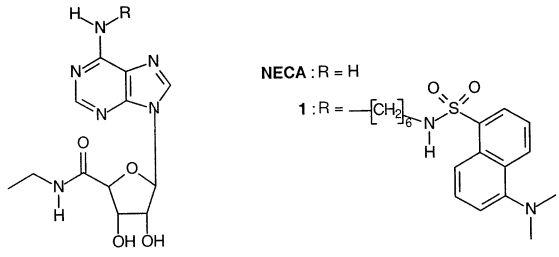
A new fluorescent ligand for adenosine receptors, obtained by the insertion of a dansylamino-moiety with a linear hexyl spacer in the N^6 position of a 1,8-naphthyridine adenosine receptor ligand, proved to possess a high affinity and selectivity for the A_1 receptor subtype.

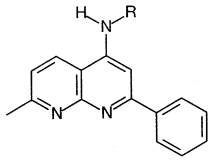
Keywords: Adenosine; A_1-selective; Fluorescent; 1,8-Naphthyridine; Dansyl

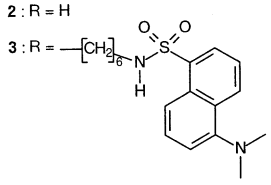
1. Introduction

The role of adenosine in many biological processes, such as platelet aggregation inhibition, blood pressure lowering, neurotransmission, etc., has been extensively demonstrated [1]. These different effects are mediated by specific receptor subtypes, which have been identified and classified as A_1, A_{2A}, A_{2B}, and A_3 [2]. The specific tissutal, cellular, and sub-cellular distribution of these receptor subtypes can be advantageously studied "in vitro" by means of fluorescent microscopy techniques using fluorescent probes, which selectively bind each receptor subtype.

We have recently reported synthesis and binding assays of NECA analogs, containing a dansylaminoalkyl moiety in the N^6 position, as A_1 selective fluorescent probes [3]. In particular, we found that the optimal chain length of the alkyl spacer, in order to get the highest affinity and selectivity for the A_1 receptor, was achieved with a C6 chain (compound **1**).







Recently, several 1,8-naphthyridine derivatives have shown excellent A_1-selective binding affinities [4]. Therefore, we envisaged the possibility of obtaining a new A_1-selective fluorescent probe, by functionalizing one of the most selective 1,8-naphthyridine derivatives, compound **2** [4], with the same dansylaminohexyl portion (C6), which already gave good results in the NECA analog **1**

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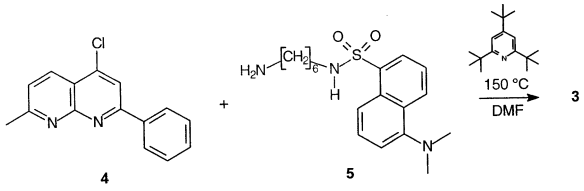
[3]. To this purpose, we synthesized fluorescent compound **3** and submitted it to A_1, A_{2A}, and A_3 adenosine receptor binding assays.

2. Chemistry

Compound **3** was prepared as reported in Scheme 1. Direct condensation of 4-chloro-7-methyl-2-phenyl-1,8-naphthyridine (**4**) [5] with mono *N*-dansylated 1,6-hexanediamine (**5**) [3] was achieved at 150 °C in DMF (16 h), in the presence of 2,4,6-tri-*tert*-butylpyridine as the acid scavenger, affording fluorescent naphthyridine derivative **3**. Compound **3** showed an excitation λ_{max} of 422 nm and an emission λ_{max} of 515 nm.

3. Results and discussion

The affinity of compounds **2** and **3** for A_1, A_2 and A_3 ARs was assessed by measuring their affinity to displace [^{3}H]CHA [6], [^{3}H]CGS 21680 [7], and [^{125}I]IB-MECA [8] binding to membranes derived from bovine cerebral cortex, bovine striatum, and CHO cells expressing human A_3 AR [9], respectively. The results of these tests are shown in Table 1.



Scheme 1.

Table 1
Affinities of derivatives **2** and **3** at A_1, A_{2A} and A_3 adenosine receptor subtypes

Comp.	K_i (A_1, nM) [a,b]	K_i (A_{2A}, nM) [a,c]	K_i (A_3, nM) [a,d]
NECA	14 ± 4 [e]	16 ± 3 [e]	36 ± 3 [f]
2	17 ± 4	420 ± 29	6220
3	13 ± 2	1430 ± 40	1560 ± 36

[a] Binding data were computer-analyzed by non-linear least-squares analysis (GraphPad Prism Softwares, San Diego, CA). IC_{50} values were determined and converted to K_i (inhibition constant) values by the Cheng and Prusoff equation [11]. Values represent the means ± SE of three experiments.

[b] Displacement of [^{3}H]CHA from bovine cortical membranes [6].

[c] Displacement of [^{3}H]CGS 21680 from bovine striatal membranes [7].

[d] Displacement of [^{125}I]IB-MECA from the human A_3 receptor [8] expressed in CHO cells [9].

[e] Ref. [4].

[f] Ref. [10].

We were pleased to find that compound **3** preserved, or even slightly improved, the good affinity (13 nM) for the A_1 receptor subtype shown by its non-fluorescent counterpart **2** (17 nM) [4]. This result confirmed the fact that the dansylated C6 alkyl chain does not disturb the interaction of the rest of the molecule with the receptor, as seen before for NECA derivative **1** [3]. Moreover, dansylated naphthyridine **3** showed an A_1/A_2 selectivity ratio which is four to five times better than the one found with non-dansylated naphthyridine **2**.

The binding properties of compound **3**, together with its structure simplicity and comfortable synthetic accessibility, makes it a promising and convenient fluorescent probe for "in vitro" localization and trafficking studies of the A_1 adenosine receptor.

4. Experimental

4.1. Chemistry

Melting points were determined on a Kofler hot-stage apparatus and are uncorrected. ^{1}H NMR spectra of all compounds were obtained with a Varian Gemini-200 instrument operating at 200 MHz; the data are reported as follows: chemical shift (in ppm) from the Me_4Si line as external standard, multiplicity (br = broad, s = singlet, d = doublet, t = triplet, m = multiplet). Mass spectra were recorded on a VG 70-250S mass spectrometer. Analytical TLCs were carried out on 0.25-mm layer silica gel plates containing a fluorescent indicator; spots were detected under UV light (254 nm). Column chromatographies were performed using 230–400 mesh silica gel (Macherey-Nagel silica gel 60 Art. no. 815381). Sodium sulfate was always used as the drying agent. Evaporations were performed in vacuo (rotating evaporator). Compound **4** [5] and compound **5** [3] were synthesized following literature procedures. Anhydrous DMF and 2,4,6-tri-*tert*-butylpyridine were purchased from Sigma-Aldrich.

4.1.1. Synthesis of 4-{6-[5-(dimethylamino)naphthalene-1-sulfonylamino]hexylamino}-7-methyl-2-phenyl-1,8-naphthyridine (3)

In a sealed vial, a solution of compound **4** (40 mg, 0.16 mmol) [5] in anhydrous DMF was treated with 61 mg (0.18 mmol) of compound **5** [3] and 60 mg of 2,4,6-tri-*tert*-butylpyridine (0.24 mmol). The resulting mixture was heated to 150 °C for 16 h. The solvent was removed under vacuum and the crude reaction product was then purified by silica gel column chromatography (dichloromethane/acetone 7:3) affording compound **3** (54% yield) as a syrup.

^{1}H NMR ($CDCl_3$, 200 MHz) δ 1.14–1.37 (m, 8H), 2.82–3.00 (m, 2H), 2.88 (s, 6H), 2.95 (s, 3H), 3.11–3.19 (m, 2H), 4.88 (t, 1H, J = 6.1 Hz), 5.69 (br, 1H), 7.16–

7.25 (m, 2H), 7.44–7.59 (m, 5H), 8.01 (br, 2H), 8.13 (s, 1H), 8.22–8.31 (m, 3H), 8.53 (d, 1H, $J = 8.4$ Hz); MS (FAB^+) *m/e* 568 $(M+H)^+$.

4.2. Biological assays

Bovine cerebral cortex and striatum membranes to be used in the competition binding assays [6–8] were prepared as reported below. After the sacrifice of the bovine, the brain was taken up and the cortex or the striatum was suspended in a 1:10 solution of 0.32 M sucrose and protease inhibitors (bacitracin 16 mg/100 ml, benzamidine 20 mg/100 ml, tripsin inhibitors 2 mg/100 ml) in a pH 7.7 buffer (Tris–HCl 50 mM, $MgCl_2$ 2 mM). The suspension was homogenized with a Ultra-turrax apparatus until complete homogenation, and then it was centrifuged at $1000 \times g$ for 5 min in a Beckman J2-21 centrifuge cooled to 4 °C. The supernatant was collected and was centrifuged again at $48\,000 \times g$ for 30 min at 4 °C. The precipitate so obtained was taken up in a cooled (4 °C) pH 7.7 buffer solution (Tris–HCl 50 mM, $MgCl_2$ 2 mM) containing protease inhibitors (bacitracin 16 mg/100 ml, benzamidine 20 mg/100 ml, tripsin inhibitors 2 mg/100 ml), and the mixture was homogenized once more with a Ultra-turrax apparatus, and eventually centrifuged at $48\,000 \times g$ for 30 min at 4 °C. The pellet so obtained was collected, taken up again with a 1:5 mixture of the same buffer/protease inhibitors solution described above and a solution containing adenosine deaminase (1 U/ml) so that the endogenous adenosine was completely removed after an incubation period of 30 min at 37 °C, under stirring. The mixture was centrifuged again at $48\,000 \times g$ for 30 min at 4 °C. The pellet was collected and divided in 0.1–0.2 g portions which were stored at −20 °C. CHO cells expressing human A_3 adenosine receptors were prepared as reported in the literature [9]. Specific binding was calculated by subtracting the non-specific binding to the total binding measured by displacement of the appropriate labeled ligand by increasing concentrations of the fluorescent probe **3**. Radioactivity measurements were done with a liquid-phase Beckman LS-1800 instrument.

4.2.1. Competition binding assay on A_1 adenosine receptors present in bovine cerebral cortex membranes

Portions (0.1 g) of bovine cerebral cortex membranes, prepared as described previously (Section 4.2), were diluted (1:20) in a pH 7.7 buffer solution (see Section 4.2) and homogenized; small portions (100 μl) of the resulting mixture were each treated with increasing concentration of fluorescent probe **3** in a 10 μl DMSO solution (the level of DMSO did not exceed 2% and was maintained constant in all tubes), 50 μl of a 1 nM solution of [^{3}H]CHA (31 Ci/mmol) and, in the case of non-specific binding measurements, also 50 μl of a 16 μM solution of R-PIA. The resulting mixtures were then diluted with the pH 7.7 buffer to a total volume of 500 μl and incubated at 25 °C for 60 min, after which time the samples were rapidly filtered under vacuum through Whatman GF/C filters, which were then washed three times with 5 ml of cold buffer. The filters were then put into plastic pony vials together with 4 ml of Beckman Ready-Protein scintillation liquid and the radioactivity was measured with a liquid-phase Beckman LS-1800 instrument.

4.2.2. Competition binding assay on A_{2A} adenosine receptors present in bovine striatum membranes

Portions (0.1 g) of bovine striatum membranes, prepared as described previously (Section 4.2), were diluted (1:20) in a pH 7.7 buffer solution (see Section 4.2) and homogenized; small portions (100 μl) of the resulting mixture were each treated with increasing concentration of fluorescent probe **3** in a 10 μl DMSO solution (the level of DMSO did not exceed 2% and was maintained constant in all tubes), 50 μl of a 5 nM solution of [^{3}H]CGS21680 (42.1 Ci/mmol) and, in the case of non-specific binding measurements, also 50 μl of a 50 μM solution of NECA. The resulting mixtures were then diluted with the pH 7.7 buffer to a total volume of 500 μl and incubated at 25 °C for 90 min, after which time the samples were rapidly filtered under vacuum through Whatman GF/C filters, which were then washed three times with 5 ml of cold buffer. The filters were treated as reported above (Section 4.2.1) for the radioactivity measurement.

4.2.3. Competition binding assay on CHO cells expressing human A_3 adenosine receptors

Portions (0.2 g) of CHO cell membranes, expressing human A_3 receptors, were diluted (1:10) in a pH 7.4 buffer solution (Tris–HCl 50 mM, EDTA 1 mM, $MgCl_2$ 10 mM) and homogenized. Small portions containing about 40 μg of proteins were treated with increasing concentration of fluorescent probe **3** in a 10 μl DMSO solution (the level of DMSO did not exceed 2% and was maintained constant in all tubes), 20 μl of a 0.2 nM solution of [^{125}I]AB-MECA (2000 Ci/mmol), 20 μl of a 2 U/ml solution of adenosine deaminase and, in the case of non-specific binding measurements, also 20 μl of a 50 μM solution of NECA. The resulting mixtures were then diluted with the pH 7.4 buffer to a total volume of 100 μl and incubated at 25 °C for 60 min, after which time the samples were rapidly filtered under vacuum through Whatman GF/C filters, which had been previously treated for 1 h at 4 °C with an aqueous solution (1 g/200 ml) of polyethylenimine (PEI). The filters were then washed three times with 5 ml of cold buffer and then treated as reported above (Section 4.2.1) for the radioactivity measurement.

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