Pergamon

Bioorganic & Medicinal Chemistry Letters 8 (1998) 3223–3228

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

FLUORESCENT PROBES FOR ADENOSINE RECEPTORS: SYNTHESIS AND BIOLOGY OF N^6-DANSYLAMINOALKYL-SUBSTITUTED NECA DERIVATIVES

Marco Macchia,[a,*] Francesca Salvetti,[b] Silvia Barontini,[a] Federico Calvani,[a] Marco Gesi,[c] Mahmoud Hamdan,[d] Antonio Lucacchini,[b] Antonio Pellegrini,[c] Paola Soldani,[c] Claudia Martini[b]

[a]*Dipartimento di Scienze Farmaceutiche and* [b]*Dipartimento di Psichiatria Neurobiologia Farmacologia e Biotecnologie, via Bonanno 6, 56126, Pisa, Italy.* [c]*Dipartimento Morfologia Umana e Biologia Applicata, via Roma 55, 56126, Pisa, Italy.* [d]*GlaxoWellcome Medicines Research Center, via Fleming 4, 37134, Verona, Italy.*

Received 3 August 1998; accepted 5 October 1998

Abstract. New fluorescent ligands for adenosine receptors are described; these compounds were obtained by the insertion, in the N^6 position of NECA (a potent adenosine agonist), of dansylaminoalkyl moieties with alkyl spacers of increasing carbon chain length (from 3 to 12). Among them, the compound with a C6 alkyl spacer proved to be the most interesting one, showing a marked selectivity for the A_1 receptor subtype; furthermore, in fluorescence microscopy assays it proved to be able to visualize and localize this receptor subtype at the level of the molecular layer of the rat cerebellar cortex.

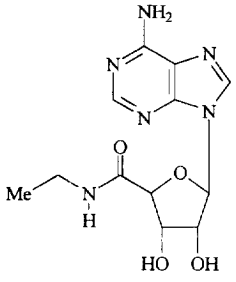
Adenosine acts as a neuromodulator in the central and peripheral nervous system and as a homeostatic regulator in a variety of other tissues, including heart, kidney and the immune system.[1] These effects are mediated by the binding of adenosine to specific receptor subtypes which have been classified as A_1 and A_2. Biochemical and functional studies have led to the identification of two A_2 receptor subtypes, A_{2a} and A_{2b}, as well as another receptor class, designated as A_3.[2] The specific regional localization of adenosine receptor subtypes in CNS and other tissues had been determined at the light microscopy level using "in vitro" receptor autoradiographic techniques.[3,4] However autoradiography does not have the resolution to determine the localization of receptors at the single cell or subcellular levels.[5]

Fluorescence microscopy is one of the most versatile techniques currently available for analyzing biomolecules and cells in view of its high sensitivity and speed; the use of specific high-affinity molecular probes could thus help to elucidate molecular characteristics of adenosine receptor subtypes, their regional distribution and cellular localization.

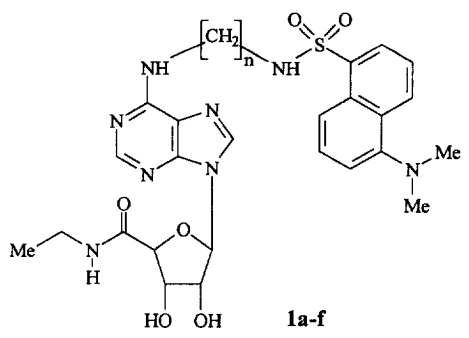
For this purpose, here we report the development of fluorescent probes for adenosine receptors (compounds **1a-f**) that are derived from NECA, a potent non-selective adenosine agonist.[6] The probes were obtained by the insertion in the N^6 position of NECA of dansylaminoalkyl moieties with alkyl spacers of increasing carbon chain length (from 3 to 12).

* E-Mail:mmacchia@farm.unipi.it. Fax: ++39-50-40517

0960-894X/98/$ - see front matter
PII: S0960-894X(98)00582-4



NECA



1a-f

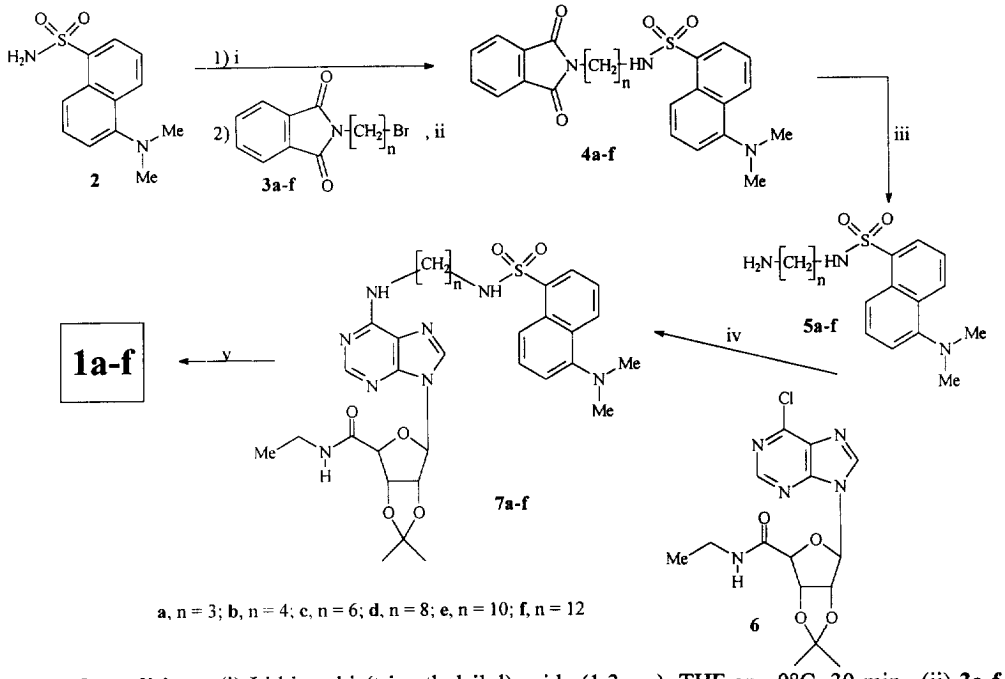
a, n = 3; **b**, n = 4; **c**, n = 6; **d**, n = 8; **e**, n = 10; **f**, n = 12

Chemistry. Compounds **1a-f** were prepared as outlined in Scheme 1. Treatment of dansylamide **2** with lithium bis(trimethylsilyl)amide in anhydrous THF at 0°C followed by alkylation with the appropriate phthalimidoalkyl bromide (**3a-f**),[7] gave the desired intermediates **4a-f** which were purified by column chromatography.[8] Subsequent hydrazinolysis of **4a-f** with hydrazine monohydrate in EtOH, yielded derivatives **5a-f**.[9] **5a-f**, after reaction with 6-chloropurine-5'-ribouronamide **6**[10] in absolute EtOH in the presence of triethylamine, gave intermediates **7a-f**, which were purified by column chromatography.[11] Lastly, the appropriate intermediate **7a-f**, by removal of the isopropylidene group using 1 N HCl, followed by neutralization at 0 °C of the resulting solution with sodium bicarbonate solution gave a white precipitate consisting almost exclusively of the appropriate final compound **1a-f**.[12]

Radioligand Binding Assays. The affinity of fluorescent probes **1a-f** for A_1, A_2 and A_3 adenosine receptor subtypes was checked by binding tests on rat cerebral cortex, rat striatal and rat testis membrane preparations, respectively. [^{3}H]-CHA and [^{3}H]-CGS 21680 were used as specific tritiated ligands for rat cortical A_1- and rat striatal A_2-receptors, respectively. [^{3}H]-(*R*)-PIA was utilized to label rat testis A_3-receptors in the presence of 150 nM DPCPX to block A_1-receptors. Membrane preparations and binding assays were carried out as previously described.[13] The results of these tests are shown in Table 1.

Compounds **1a-c** (with n varying from 3 to 6) showed a good selective binding affinity for the A_1 receptor subtype; in particular, fluorescent probe **1c** (n = 6) seemed to be the most interesting one, with a K_i for the A_1 receptor of 27 nM against K_i values for the A_2 receptor of 4300 nM and for the A_3 receptor of 3600 nM.

Scheme 1



Reagents and conditions. (i) Lithium bis(trimethylsilyl)amide (1.3 eq.), THF an., 0°C, 30 min.. (ii) **3a-f** (1.3 eq.), THF an., r.t. for 18h then reflux for 10h; yields from 19% to 35%. (iii) Hydrazine monohydrate (2 eq.), EtOH, 45°C, 24h; yields from 66% to 88%. (iv) **6** (0.9 eq.), triethylamine (2 eq.), abs. EtOH, 75°C, 18h; yields from 52% to 92%. (v) 1N HCl (10 eq.), 60°C, 4h., then $NaHCO_3$ solution at 0 °C until pH = 7; yields from 40% to 77%.

Compound **1d** also possessed an appreciable affinity for the A_1 receptor subtype, a decidedly lower affinity for A_2 receptors, and the best affinity for A_3 receptors, thus losing the selectivity for A_1 receptors shown by derivatives **1a-c**. Compounds **1e,f** possessed a low affinity for all the three receptor subtypes.

A comparison of binding affinity data for A_1 receptors of fluorescent probes **1a-f** with the various alkyl spacers inserted between the dansylamino moiety and the N^6 amino group seems to indicate that shorter spacers (varying from n = 3 to n = 6) are preferred to longer ones (from n = 8 to n = 12); in particular, a C6 alkyl spacer (compound **1c**) seems to be the most preferred one for interaction with the A_1 receptor subtype. Furthermore, none of the alkyl spacers present in compounds **1a-f** proved to be able to allow the right interaction of these

types of structures with A_2 receptors. As regards A_3 receptors, only a C8 alkyl spacer, as in compounds **1d**, seemed to be tolerated.

Table 1. Radioligand Binding Affinity of Fluorescent Probes **1a-f**.

Compd	n	K_i[a] (A_1, nM)	K_i (A_2, nM)	K_i (A_3, nM)
1a	3	42 ± 4	4300 ± 300	1800 ± 100
1b	4	70 ± 6	9500 ± 600	2000 ± 150
1c	6	27 ± 3	4300 ± 400	3600 ± 260
1d	8	111 ± 13	>10000	252 ± 18
1e	10	234 ± 20	>10000	1100 ± 100
1f	12	3400 ± 200	>10000	2500 ± 250

[a]Binding data were computer-analyzed by non-linear least squares analysis (GraphPad Prism Softwares, San Diego, CA). IC_{50} values determined and converted to K_i (inhibition constant) values by the Cheng and Prusoff equation.[14] Values represent the means ± S.E. of three experiments.

Fluorescence Microscopy Assays. Sections of rat cerebellar cortex, whose molecular layer has previously been reported to possess a high density of the A_1 receptor subtype,[4] were incubated with compound **1c**, i.e the most active and selective one for the A_1 receptor subtype, and observed with a fluorescence microscope.[15] As can be seen from Figure 1, controls did not show any fluorescence, while non-specific (Figure 2) and total binding (Figure 3) showed a significant difference in fluorescence at the level of the molecular layer of the cerebellar cortex. Compound **1c** thus proved to be capable of visualizing and localizing A_1 receptor subtype in these tissue sections.

Conclusions. Compounds **1a-f**, obtained by the insertion in the N^6 position of NECA, of dansylaminoalkyl moieties with alkyl spacers of increasing carbon chain length (from 3 to 12), were designed as new fluorescent probes for adenosine receptors; among them, compound **1c** proved to be the most interesting one, as it showed a marked selectivity for the A_1 receptor subtype. Furthermore, fluorescence microscopy assays proved that compound **1c** was able to visualize and localize the A_1 receptor subtype at the level of the molecular layer of the rat cerebellar cortex. For these reasons, compound **1c** may be considered as a new fluorescent ligand for the study of the A_1 receptor subtype.

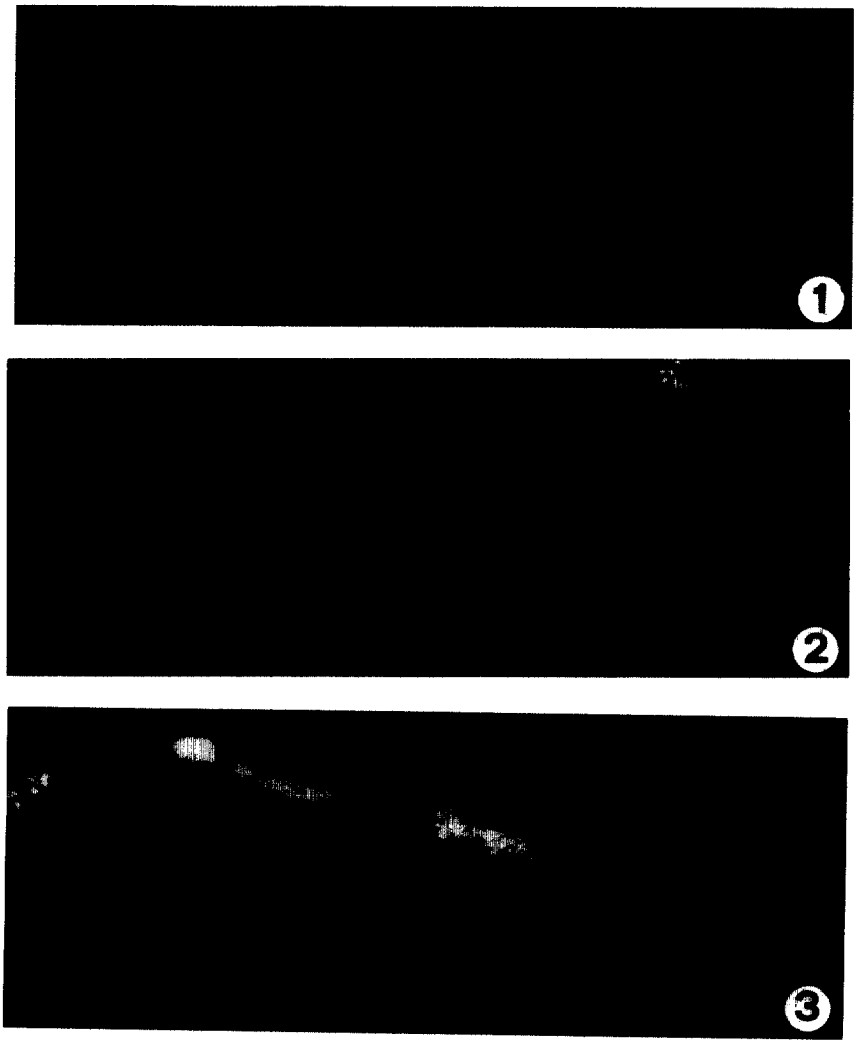


Figure 1. *Control*. Cryo-section of rat cerebellar cortex without incubation with fluorescent ligand; x150.

Figure 2. *Non-specific binding*. Cryo-section of rat cerebellar cortex incubated with compound **1c** (100 nM) and (*R*)-PIA (1.5 μM). At this concentration (*R*)-PIA was able to block A_1 receptors; x150.

Figure 3. *Total binding*. Cryo-section of rat cerebellar cortex incubated with compound **1c** (100 nM); x150.

References and Notes

1. Daval, J. L.; Nehlig, A.; Nicolas, F. *Life Sciences* **1991**, *49*, 1435.
2. Fredholm, B. B.; Abbracchio, M. P.; Burnstock, G.; Daly, J. W.; Kendall Harden, T.; Jacobson, K. A.; Leff, P.; Williams, M. *Pharmacol. Rev.* **1994**, *46*, 143.
3. Jarvis, M. F.; Jacobson, K. A.; Williams, M. *Neuroscience Letters* **1987**, *81*, 69.
4. Weber, R. G.; Jones, R. C.; Palacios, J. M.; Lohse, M. J. *Neuroscience Letters* **1988**, *87*, 215.
5. McGrath, J. C.; Arribas, S.; Daly, C. J. *TiPS* **1996**, *17*, 393.
6. Bruns R.F. In *Adenosine Receptors*; Cooper, D.M.F.; Londos, C. Ed.; Alan R. Liss: New York, 1988; pp. 54-63.
7. Compounds **3a,b** were commercially available (Aldrich). Compounds **3c-f** were prepared as previously reported (Alisi, M. A.; Brufani, M.; Filocamo, L.; Gostoli, G.; Licandro, E.; Cesta, M. C.; Lappa, S.; Marchesini, D.; Pagella, P. *Bioorg. & Med. Chem. Letters* **1995**, *5*, 2077).
8. For example: Compound **4a**: ^{1}H-NMR ($CDCl_3$, 80 MHz) δ 8.53-7.09 (m, 10 H), 3.69-3.51 (m, 2H), 3.11-2.86 (m, 8 H), 1.81-1,57 (m, 2 H); MS m/e 437 (M^+).
9. After 24 h at 45 °C, the solvent was evaporated and the residue was then dissolved in EtOAc and washed with 3% aqueous K_2CO_3 solution. The organic phase was then dried ($MgSO_4$) and evaporated to give the appropriate intermediate **5a-f**, which was used without further purification in the next step. For example: Compound **5a** (Abdel-Monem, M. M.; Ohno, K. *J. Pharm. Sci.* **1977**, *66*, 1089): ^{1}H-NMR ($CDCl_3$, 80 MHz) δ 8.53-7.08 (m, 6 H), 3.11-2.87 (m, 8 H), 2.67-2.50 (m, 2H), 1.83-1,58 (m, 2 H); MS m/e 307 (M^+).
10. Olsson, R. A.; Kusachi, S. K.; Thompson, R. D.; Ukena, D; Padgett, W.; Daly, J. W. *J. Med. Chem.* **1986**, *29*, 1683.
11. For example: Compound **7a**: ^{1}H-NMR ($CDCl_3$, 80 MHz) δ 8.53-7.08 (m, 8 H), 6.10 (s, 1 H), 5.35-5.24 (m, 2 H), 4.66 (s, 1 H), 3.68-3.53 (m, 2 H), 3.25-2.85 (m, 10 H), 1.82-1,56 (m, 2 H), 1.53 (s, 3 H), 1.35 (s, 3 H), 1.04 (t, J = 7.2 Hz, 3H); MS (FAB^+) m/e 639 $(M + H)^+$.
12. For example: Compound **1a**: ^{1}H-NMR (DMSO-d6, 80 MHz) δ 8.54-7.07 (m, 8 H), 5.98 (d, J = 7.6 Hz, 1H), 5.72 (d, J = 4.2 Hz, 1 H, OH), 5.51 (d, J = 6.3 Hz, 1 H, OH), 4.69-4.55 (m, 1 H), 4.34 (s, 1 H), 4.26-4.15 (m, 1 H), 3.72-3.56 (m, 2 H), 3.28-2.83 (m, 10 H), 1.83-1.57 (m, 2 H), 1.09 (t, J = 7.2 Hz, 3H); MS (FAB^+) m/e 599 $(M + H)^+$.
13. Franchetti, P.; Cappellacci, L.; Marchetti, S.; Trincavelli, L.; Martini, C.; Mazzoni, M. R.; Lucacchini, A.; Grifantini, M. *J. Med. Chem.* **1998**, *41*, 1708.
14. Cheng, Y. C.; Prusoff, W.H. *Biochem. Pharmacol.* **1973**, *22*, 3099.
15. *Control*: Cryo-sections of rat cerebellar cortex (20μm) were incubated with buffer A [PBS buffer pH 7.5 with (mg/100ml) 20% bacitracin (Fluka Chemie AG, Buchs, Switzerland), 16% benzamidine, 2% inhibitors of trypsin, 2 U/ml adenosine deaminase] and 1.5 μM (*R*)-PIA (Sigma Chemical Co., St. Louis MO, USA) for 20 min. and then with buffer B [PBS buffer pH 7.5 with 2 mM $MgCl_2$] for 2 h. *Non-specific binding*: cryo-sections of rat cerebellar cortex (20μm) were incubated with buffer A with 1.5 μM (*R*)-PIA for 20 min., and then with compound **1c** (100 nM) in buffer B and 1.5 μM (*R*)-PIA for 2 h. *Total binding*: cryo-sections of rat cerebellar cortex (20μm) were incubated with buffer A for 20 min., and then with compound **1c** (100 nM) in buffer B for 2 h.
    The sections were observed using an Orthoplan Leitz (Wetzlar) fluorescence microscope.