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7-Nitrobenzofurazan (NBD) Derivatives of 5′-*N*-Ethylcarboxamidoadenosine (NECA) as New Fluorescent Probes for Human A_3 Adenosine Receptors

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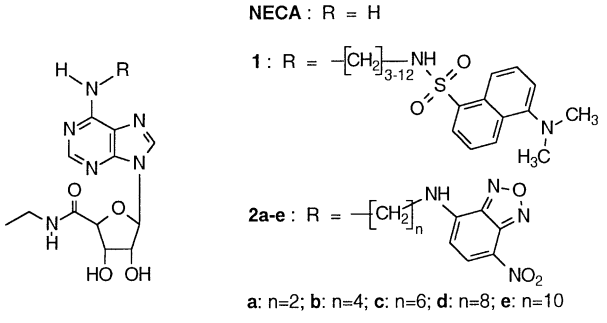
Abstract—New fluorescent ligands for adenosine receptors (ARs), obtained by the insertion, in the N^6 position of NECA, of NBD-moieties with linear alkyl spacers of increasing length, proved to possess a high affinity and selectivity for the A_3 subtype expressed in CHO cells. In fluorescence microscopy assays, compound **2d**, the most active and selective for human A_3-AR, permitted visualization and localization of this human receptor subtype, showing its potential suitability for internalization and trafficking studies in living cells. © 2001 Elsevier Science Ltd. All rights reserved.

Adenosine is an autacoid that exerts its physiological actions through specific cell surface receptors. The pharmacological, behavioral and binding properties of adenosine receptors (ARs) have been extensively studied.[1] By comparison, much less is known about the intracellular routing and addressing of ARs either before or after agonist binding. Yet an understanding of the cellular regulation of this class of receptors is of importance, since it is involved in the mechanism of receptor desensitization, resensitization and down regulation.[2,3] Unfortunately, the main limitation of receptor internalization and trafficking studies is related to the protocol, which requires fixing of the cells and prohibits a direct correlation between receptor activation, trafficking and desensitization in living cells. One way to overcome this obstacle would be to probe the ligand–receptor complex with a fluorescently labeled agonist able to be further visualized in living cells by means of confocal microscopy. Past attempts to develop AR ligands for this purpose were made by K. A. Jacobson, using new macromolecular probes in which the avidin–biotin technology was applied.[4]



We have recently developed and characterized compounds of type **1** as fluorescent ligands for ARs. These probes were obtained by the insertion, in the N^6 position of NECA, a potent adenosine agonist, of dansylaminoalkyl

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moieties with alkyl spacers of increasing linear aliphatic chain length (from 3 to 12 carbon atoms).[5] Although the compounds have been successfully employed to visualize A_1 ARs in rat cerebellar cortex by fluorescence microscopy, their use is restricted by the short fluorophore excitation wavelength (340 nm) at which many cells and tissues autofluoresce. To overcome these problems, we thought it conceivable to prepare new fluorescent ligands, in which the dansyl group of compounds of type **1** has been replaced by a longer excitation wavelength fluorophore such as 7-nitrobenzofurazan (NBD, Abs 465 nm, Em 535 nm).

Herein, we describe the development and characterization of fluorescent compounds **2a–e** and their utility in visualizing adenosine receptors.

Chemistry

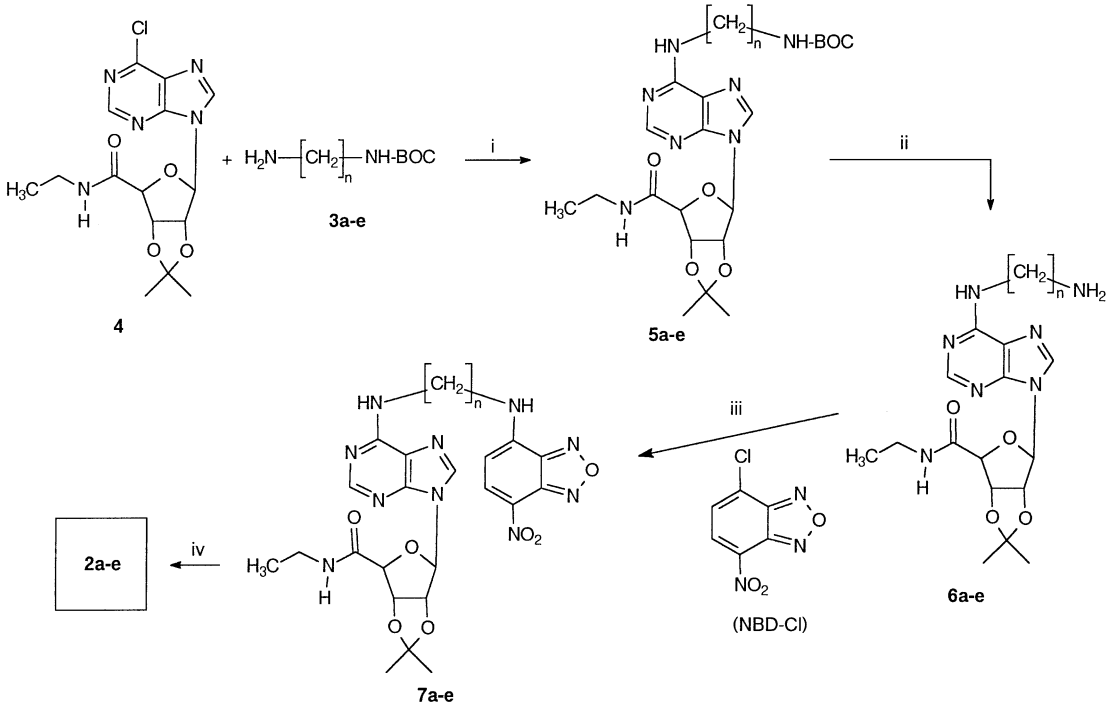
Compounds **2a–e** were prepared as outlined in Scheme 1. Treatment of 6-chloropurine-5′-ribouronamide **4**[6] with the appropriate mono *N*-Boc-protected diamine **3a–e** afforded purine intermediates **5a–e**, which were purified by column chromatography.[7] Compounds **3a–c** were commercially available, whereas compounds **3d** and **3e** were prepared by mono-Boc protection of 1,8-diaminooctane and 1,10-diaminodecane, respectively. Subsequent deprotection of the *N*-Boc group present in **5a–e** with trifluoroacetic acid in CH_2Cl_2 afforded free primary amines **6a–e** which were directly used in the next step. The condensation between purines **6a–e** and 7-nitrobenzo-2-oxa-1,3-diazole chloride (NBD-Cl) was run in anhydrous THF, in the presence of 2,6-di-*t*-butyl-4-methylpyridine (DTBMP) as the 'acid scavenger' base, affording NBD-substituted purines **7a–e**.[8] Final deprotection of the acetonide group present in the sugar portion of **7a–e** was achieved by acid hydrolysis with a 1 N aqueous solution of hydrochloric acid, affording, after column chromatography purification, the target adenosine fluorescent probes **2a–e**.[9]

Radioligand Binding Assays

The affinity of fluorescent probes **2a–e** for A_1, A_{2a} and A_3 ARs was assessed by measuring their ability to displace [^{3}H]DPCPX,[10,11] [^{3}H]CGS 21680,[12,13] and [^{125}I]AB-MECA[14] binding to membranes derived from human cerebral cortex, human striatum and CHO cells[15] expressing human A_3 ARs, respectively.[16] The results of these tests are shown in Table 1. Compounds **2a–e** generally showed a good binding affinity for the A_3 receptor subtype; in particular, fluorescent probes **2c–e** (with *n* varying from 6 to 10) seemed to be the most interesting ones, with K_i values for the A_3 receptor ranging from 7.4 to 28.1 nM.

In contrast, all the compounds synthesized possess a low affinity for the A_1 receptor subtype, especially in the case of compounds **2a–c** (with *n* varying from 2 to 6) whose K_i values were higher than 10 μM.

Also, in the case of the A_{2a} receptor subtype, the affinities of compounds **2a–e** were modest, with K_i values in the micromolar range. Among all the compounds tested, fluorescent probe **2d** ($n=8$) proved to be the most active and selective one for the A_3 receptor subtype, with an affinity about 450 and 680 times higher than that for the A_1 and A_2 receptor subtypes, respectively.



Scheme 1. Reagents and conditions: (i) triethylamine, abs EtOH, 80 °C, 24 h; yields from 32 to 94%; (ii) CF_3COOH/CH_2Cl_2 (1:1), rt, 2 h; (iii) DTBMP, an THF, 70 °C, 48 h; yields from 21 to 42%, two steps; (iv) 1 N HCl, 60 °C, 6 h; yields from 25 to 58%.

Table 1. Affinities of derivatives **2a–e** at human A_1, A_{2a} and A_3 adenosine receptor subtypes

Compd	*n*	K_i (A_1, nM)[a,b]	K_i (A_{2a}, nM) [a,c]	K_i (A_3, nM) [a,d]
2a	2	>10,000	6750±1021	55.8±3.44
2b	4	>10,000	>10,000	381±85.7
2c	6	>10,000	6083±912	23.6±3.25
2d	8	3476±521	5096±764	7.44±2.38
2e	10	6350±952	6100±915	28.1±3.80
NECA		200±45.3	15.1±3.42	8.22±2.14

[a]Binding data were computer-analyzed by non-linear least squares analysis (GraphPad Prism Softwares, San Diego, CA, USA). IC_{50} values were determined and converted to K_i (inhibition constant) values by the Cheng and Prusoff equation.[17] Values represent the means±SE of three experiments.
[b]Displacement of [^{3}H]DPCPX from human cortical membranes.[10,11]
[c]Displacement of [^{3}H]CGS 21680 from human striatal membranes.[12,13]
[d]Displacement of [^{125}I]AB-MECA from the human A_3 receptor[14] expressed in CHO cells.[15]

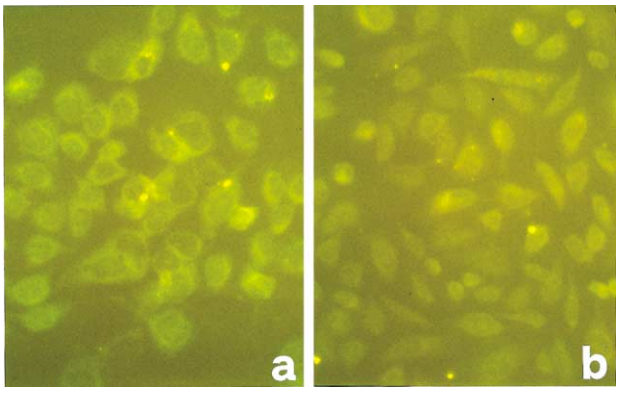


Figure 1. Fluorescent labeling studies of human A_3 ARs in CHO cells. CHO cells were cultured on coverslips and incubated with compound **2d** (500 nM) for 120 min at 22 °C in the (a) absence or (b) presence of 500 μM of NECA. At the end of the incubation time, the cells were washed, and A_3 ARs were localized by fluorescence microscopy. Magnification ×335.

Fluorescence Microscopy Assays

Compound **2d**, the most active and selective one for the A_3 receptor subtype, was incubated for 2 h with CHO cells expressing human A_3 ARs.[15]

This incubation time was established based on preliminary experiments in which we observed that cellular fluorescence was visible after 1 h incubation time and reached the saturation point at around 2 h. After rapid washing, the cells were observed with a fluorescence microscope.[18] At a concentration about 50 times higher than the K_i value (necessary to obtain a consistent, reproducible labeling), compound **2d** (500 nM) selectively stained almost exclusively the membrane surface of CHO cells expressing A_3 ARs (see Fig. 1a). Binding was completely prevented in the presence of the adenosine agonist NECA (500 μM) (see Fig. 1b).

Discussion and Conclusions

We synthesized compounds **2a–e** as a new class of fluorescent derivatives. These were obtained by the insertion of NBD with alkyl spacers of increasing carbon chain length in the N^6 position of NECA. This structural modification confers to **2a–e** a pharmacological profile which is different from NECA. These derivatives generally possess a high affinity and selectivity for the A_3 receptor subtype expressed in CHO cells, with no significant affinity for A_1 or A_{2a} human ARs.

All the alkyl spacers allow an efficient and selective interaction of these types of structures with A_3 receptors, although the longer ones (**2c–e**, $n=6$–10) seem to be preferred.

Compound **2d** proved to be the most active and selective for human A_3 ARs and it was then used in fluorescence microscopy. Real-time visualization of binding of the fluorescent compound was successfully achieved in CHO cells transfected with human A_3 ARs. The fluorescent staining of the plasma membrane was almost completely inhibited by an excess of NECA, demonstrating the specificity of the interaction with A_3 ARs.

In conclusion, compound **2d** can be considered as a new selective fluorescent probe for human A_3 receptor subtype. This new probe, possessing a 7-nitrobenzofurazyl fluorophore which is excited by visible light (Abs 465 nm) is suitable to visualize and localize human A_3 ARs in cells. This property of compound **2d** makes it a potentially useful probe for receptor internalization and trafficking studies in living cells.

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7. For example, compound **5d**: ^{1}H NMR ($CDCl_3$, 200 MHz) δ 0.91 (t, 3H, $J=7.3$ Hz), 1.38 (s, 3H), 1.44 (s, 9H), 1.62 (s, 3H), 1.27–2.80 (m, 12H), 3.04–3.21 (m, 4H), 3.49–3.62 (m, 2H), 4.52 (bs, 1H, NH), 4.71 (d, 1H, $J=1.5$ Hz), 5.30–5.40 (m, 2H), 5.86 (bs, 1H, NH), 6.02 (d, 1H, $J=2.5$ Hz), 7.19 (bs, 1H, NH), 7.79 (s, 1H), 8.32 (s, 1H); MS (FAB^+) *m/e* 576 $(M+H)^+$.
8. For example, compound **7d**: ^{1}H NMR ($CDCl_3$, 200 MHz) δ 0.91 (t, 3H, $J=7.4$ Hz), 1.39 (s, 3H), 1.63 (s, 3H), 1.39–1.98 (m, 12H), 3.07–3.21 (m, 2H), 3.43–3.74 (m, 4H), 4.71 (s, 1H), 5.29–5.36 (m, 2H), 6.02–6.05 (m, 1H), 6.18 (d, 1H, $J=8.8$ Hz), 6.33 (bs, 1H, NH), 7.83 (s, 1H), 8.32 (s, 1H), 8.51 (d, 1H, $J=8.3$ Hz); MS (FAB^+) *m/e* 639 $(M+H)^+$.
9. For example, compound **2d**: ^{1}H NMR (CD_3OD, 200 MHz) δ 0.62 (t, 3H, $J=7.1$ Hz), 1.16–1.76 (m, 12H), 3.29–3.63 (m, 6H), 4.48 (s, 1H), 5.14–5.21 (m, 2H), 6.02 (d, 1H, $J=7.9$ Hz), 6.34 (d, 1H, $J=8.3$ Hz), 8.14 (s, 1H), 8.21 (s, 1H), 8.52 (d, 1H, $J=8.5$ Hz); MS (FAB^+) *m/e* 599 $(M+H)^+$.

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