Bioorganic & Medicinal Chemistry Letters 23 (2013) 26–36

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

BMCL digest

Fluorescent ligands for adenosine receptors

Eszter Kozma [a], P. Suresh Jayasekara [a], Lucia Squarcialupi [a], Silvia Paoletta [a], Stefano Moro [b], Stephanie Federico [c], Giampiero Spalluto [c], Kenneth A. Jacobson [a,*]

[a] Molecular Recognition Section, Laboratory of Bioorganic Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892-0810, USA
[b] Molecular Modeling Section (MMS), Dipartimento di Scienze del Farmaco, Università di Padova, via Marzolo 5, I-35131 Padova, Italy
[c] Dipartimento di Scienze Chimiche e Farmaceutiche, Università di Trieste, Piazzale Europa 1, I-34127 Trieste, Italy

ARTICLE INFO

Article history:
Received 15 August 2012
Revised 5 October 2012
Accepted 28 October 2012
Available online 5 November 2012

Keywords:
Purine
G protein-coupled receptor
Fluorescence
Membrane proteins
Binding
Screening

ABSTRACT

Interest is increasing in developing fluorescent ligands for characterization of adenosine receptors (ARs), which hold a promise of usefulness in the drug discovery process. The size of a strategically labeled AR ligand can be greatly increased after the attachment of a fluorophore. The choice of dye moiety (e.g. Alexa Fluor 488), attachment point and linker length can alter the selectivity and potency of the parent molecule. Fluorescent derivatives of adenosine agonists and antagonists (e.g. XAC and other heterocyclic antagonist scaffolds) have been synthesized and characterized pharmacologically. Some are useful AR probes for flow cytometry, fluorescence correlation spectroscopy, fluorescence microscopy, fluorescence polarization, fluorescence resonance energy transfer, and scanning confocal microscopy. Thus, the approach of fluorescent labeled GPCR ligands, including those for ARs, is a growing dynamic research field.

Published by Elsevier Ltd.

Fluorescent ligands have been used since the 60's, however they have been used mainly as histological stains.[1] The first report on fluorescent labeling with a selective small molecule ligand of a G protein-coupled receptor (GPCR) targeted the β-adrenergic receptors in 1976.[2] In this case, an antagonist propranolol was conjugated to a 9-aminoacridine fluorophore to generate 9-AAP, which was then used to label β-receptors in rat cerebellum. Many other fluorescent GPCR ligands have followed. Thus, receptor-selective fluorescent ligands are useful tools for studying receptor physiology or pathophysiology on the cellular or even subcellular level. These fluorescent ligands provide a better understanding of receptor location, function and regulation.[3,4] Moreover, when using fluorescent dyes with different spectral characteristics (multilabeling), receptor colocalization can be detected.[5]

Fluorescent ligands have been increasingly investigated for use in drug discovery to replace the radioligands, which have served as pharmacological tools for decades.[6,7] Recently, the expense, health risks, and disposal issues that radioligands pose have increased the need to develop more efficient techniques. Fluorescent methods solve many of these issues associated with radioactivity, in addition to providing other advantages. Fluorescence allows the real-time visualization of the labeled receptor, and therefore dynamic changes like agonist-stimulated receptor internalization, endocytosis and recycling can be followed.[8] Several fluorescent techniques are used in drug discovery, such as scanning confocal microscopy (SCM), fluorescence polarization (FP), fluorescence correlation spectroscopy (FCS), fluorescence resonance energy transfer (FRET) and flow cytometry (FCM).[9,10] They allow the development of novel techniques using selective fluorescent ligands, which are comparable, and sometimes even superior, to the methods utilizing radioligands at the cellular or subcellular level.

There are three major design considerations for each target receptor, ligand class, and mode of use of the fluorescent conjugate: the choice of proper pharmacophore, fluorophore and the optimization of the linker length between them. A variety of wavelengths,

Abbreviations: AR, adenosine receptor; cAMP, cyclic adenosine 3′,5′-monophosphate; BODIPY, boron-dipyrromethene; CFP, cyan fluorescent protein; CHO, Chinese hamster ovary; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; FCM, flow cytometry; FCS, fluorescence correlation spectroscopy; FITC, fluorescein isothiocyanate; FLAG, DYKDDDDK (octapeptide); FM, fluorescence microscopy; FP, fluorescence polarization; FRET, fluorescence resonance energy transfer; GPCR, G protein-coupled receptor; HBSS, Hank's buffered salt solution; NECA, 1-(6-amino-9*H*-purin-9-yl)-1-deoxy-*N*-ethyl-β-D-ribofuranuronamide; NIR, near infrared; MRS1220, *N*-[9-chloro-2-(2-furanyl)[1,2,4]triazolo[1,5-*c*]quinazolin-5-yl]benzeneacetamide; NBD, 7-nitrobenzofurazan-4-yl; PAMAM, polyamidoamine; PTP, pyrazolo[4,3-*e*][1,2,4]triazolo[1,5-*c*]pyrimidin-5-amine; SCM, scanning confocal microscopy; TAMRA, carboxytetramethylrhodamine; TQO, [1,2,4]triazolo[4,3-*a*]quinoxalin-1-one; TQZ, [1,2,4]triazolo[1,5-*c*]quinazolin-5-amine; XAC, 8-[4-[[[[(2-aminoethyl)amino]carbonyl]methyl)oxy]phenyl]-1,3-dipropylxanthine.

* Corresponding author. Address: Molecular Recognition Section, Laboratory of Bioorganic Chemistry, Bldg. 8A, Rm. B1A-19, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892, USA. Tel.: +1 301 496 9024; fax: +1 301 480 8422.
E-mail address: kajacobs@helix.nih.gov (K.A. Jacobson).

0960-894X/$ - see front matter Published by Elsevier Ltd.
http://dx.doi.org/10.1016/j.bmcl.2012.10.112

molecular weights, polarity, charge, quantum yield, and stability of the fluorescent signal are available in chemically functionalized fluorescent dyes. The proper fluorophore requires suitable properties of fluorescence intensity, photobleaching and absorption and emission maxima for the fluorescence method chosen. In general, dyes with the lowest photobleaching rate and the highest fluorescence intensity are preferred. Nowadays, fluorophores with emission in the near infrared spectrum (NIR) are preferred, e.g. borondipyrromethene (BODIPY) 630/650 or Cy5, since their fluorescence emission can be clearly distinguished from the emission of the amino acids of living cells (autofluorescence). It is also preferred to choose dyes with high Stokes-shift (difference between the absorption and emission maxima), since the excitation light cannot interfere with the fluorescence emission giving false values. There are several other considerations, e.g. the membrane permeability (in whole cell assays) and the fluorescence lifetime (in FP) of the dye moiety. There is not a general dye useful in all fluorescent experiments, and therefore there is a need to develop alternative dyes. Additionally, a careful choice of the fluorophore for each type of experiment is indispensable. It is also essential to properly choose a pharmacophore that is potent, selective and functionalized for coupling with a linker. The length and chemical properties of this linker determine the position of the fluorophore when bound to the receptor. Moreover, the choice of dye moiety, attachment point and linker length can greatly alter the selectivity and potency of the parent pharmacophore moiety, even to the degree of reversing receptor subtype selectivity. Molecular modeling informed by the recent elucidation of GPCR X-ray structures is of increasing utility in both explaining empirical observations with new fluorescent ligands and in predicting a likely structural approach for the design of new fluorescent conjugates.

There are several comprehensive reviews about selective fluorescent ligands of different receptor families,[11–13] including GPCRs,[14,15] which are the largest single protein class of pharmaceutical targets. A protocol to study GPCR binding with fluorescent ligands in SCM studies and FCS experiments was reported by Briddon et al.[16] In considering the growing number of published fluorescent ligands for each type of receptor, it is necessary to review each GPCR family and their related fluorescent ligands. Herein, this paper reviews the fluorescent ligands selectively designed for adenosine receptors (ARs).

ARs represent a pharmaceutically important class of GPCRs involved in nearly every physiological system in the body.[17] The four subtypes include: A_1 and A_3 ARs, which inhibit production of adenosine cyclic 3′,5′-monophosphate (cAMP), and A_{2A} and A_{2B} ARs, which stimulate the production of cAMP. Nearly all known AR agonists are nucleoside derivatives, and many classes of planar heterocycles, such as naturally occurring alkylxanthines, serve as AR antagonists.

First fluorescent AR ligands

The first examples of fluorescent AR ligands were compounds **1**, **2**, **37** and **38** (structures in Table 1 and Fig. 1, receptor affinity in Table 2) synthesized by Jacobson et al. in 1987.[18] Strategically functionalized congeners of known GPCR agonists and antagonists were explored as a general approach to molecular probes for the receptors.[19] Among the amine derivatives designed as antagonist probes of the ARs, a xanthine amine congener (XAC) was devised by chain attachment of a 1,3-dialkylxanthine through a substituted 8-phenyl ring having an electron donating para-ether substituent. Agonist (N^6-modified adenosines) and antagonist (C8-modified alkylxanthines) ligands with high affinities were coupled to chemically-reactive fluorescent dye derivatives (fluorescein isothiocyanate—FITC and 7-nitrobenzofurazan-4-yl—NBD) with retention, in some cases, of high A_1AR affinity. This demonstrated the feasibility of using a tethering approach for AR fluorescent ligands. Conjugation of the functionalized congeners with biotin provided an additional strategy for receptor-labeling.[20] A subsequent study focused on the A_{2A}AR and revealed that a C2-modified adenosine derivative of FITC (FITC-APEC, **3**) could be used as a tracer in binding experiments in bovine brain membranes from the striatal region high in A_{2A}AR.[21]

Another early example of fluorescent AR ligands is from Macchia et al.[22,23] In 1998, a series of fluorescent derivatives of the widely used potent AR agonist 5′-*N*-ethylcarboxamidoadenosine (NECA) were synthesized by the insertion of dansyl-aminoalkyl moieties to the N^6-position with linear alkyl spacers of increasing carbon chain length (**6**–**11**).[22] Compound **8** was used to visualize the A_1AR in the rat cerebellar cortex by fluorescence microscopy (FM). However, the use of dansyl derivatives was limited due to the short excitation wavelength of the fluorophore (340 nm). To overcome this problem, five fluorescent derivatives were synthesized by the attachment of a fluorescent NBD moiety (excitation at 535 nm) to an AR agonist pharmacophore (**14**–**18**).[23] The fluorescent derivatives showed high affinity toward hA_3AR (Table 2). The most potent NBD derivative in the series **17** (500 nM, incubation for 120 min at 22 °C) was used to label A_3ARs expressed in CHO cells (CHO-A_3AR cells) using FM, and it selectively and specifically labeled the receptor on the membrane surface.

The above compounds **6**–**18** in the Macchia et al. study were the first examples of fluorescent AR ligands in which the choice of the fluorescent dye largely influenced the AR subtype selectivity.[22,23] For example, the dansyl-derivatives (**6**–**11**) showed modest selectivity toward the A_1AR, while the NBD ligands (**14**–**18**) were selective for the A_3AR. This study also demonstrated a dependence of the AR potency on the length of the linker between the dye and the pharmacophore.

BODIPY 630/650-X derivative AR agonists

Middleton et al. added five red-emitting BODIPY 630/650-X labeled derivatives of adenosine and NECA to the growing list of fluorescent AR ligands (**36**, **19**–**22**).[24] BODIPY630/650 was chosen due to its high fluorescent intensity, low photobleaching rate and the fact that a relatively small amount of dye is in triplet state.[25] "X" refers to an ε-aminocaproyl linker. Similarly to Macchia et al., a commercially available fluorescent dye was introduced at the N^6-position through linkers of different lengths. In whole cell binding assays using the high affinity A_1AR antagonist [^{3}H]DPCPX, the binding affinities of **19**, **20** and **36** were similar (pK_D = 6.61, 6.80 and 6.65, respectively) (Table 2). However, in [^{3}H]cAMP accumulation assays these compounds displayed different potencies at the A_1AR (pEC_{50} = 9.16, 8.47 and 8.29, respectively). ABEA-X-BY630 (**20**) displayed high potency at G_i-protein coupled A_1AR and A_3AR (pEC_{50} = 8.47 and 8.57, respectively) and lower potency at G_s-coupled A_{2A}AR and A_{2B}AR (pEC_{50} = 6.76 and 5.69, respectively). These results clearly demonstrated the dependence of functional potency on the linker structure, which was not evident in the binding affinity.

Compounds **36** and **19**–**22** were used to microscopically visualize ligand-receptor interactions.[24] The binding of these fluorescent compounds was specific and restricted to the cell membrane of CHO-A_1AR cells (100 nM, 5 min, 22 °C). Furthermore, co-localization assays were included, using CHO cells expressing an A_1AR fused on its C-terminus to Topaz (Tpz) fluorescent protein (CHO-A_1AR-Tpz cells). The authors showed that the intensity of fluorescent labeling was dependent on the linker length, with compounds **20** and **21** showing the highest and **22** showing the lowest levels of receptor binding (chains of 3, 4 and 8 atoms, respectively) in agreement with the affinity values.