Synthesis of novel (benzimidazolyl)isoquinolinols and evaluation as adenosine A1 receptor tools†

Sameek Singh,a Samantha L. Cooper,b Jacqueline R. Glenn,b Jessica Beresford,b Lydia R. Percival,b Joel D. A. Tyndall, Stephen J. Hill,a Laura E. Kilpatrickb and Andrea J. Vernallb,a*

G protein-coupled receptors (GPCRs) constitute the largest family of transmembrane receptors in eukaryotes. The adenosine A1 receptor (A1AR) is a class A GPCR that is of interest as a therapeutic target particularly in the treatment of cardiovascular disease and neuropathic pain. Increased knowledge of the role A1AR plays in mediating these pathophysiological processes will help realise the therapeutic potential of this receptor. There is a lack of enabling tools such as selective fluorescent probes to study A1AR, therefore we designed a series of (benzimidazolyl)isoquinolinols conjugated to a fluorescent dye (31–35, 42–43). An improved procedure for the synthesis of isoquinolinols from tetrahydroisoquinolinols via oxidation with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDO) and atmospheric oxygen is reported. This synthetic method offers advantages over previous metal-based methods for the preparation of isoquinolinols and isoquinolines, which are important scaffolds found in many biologically active compounds and natural products. We report the first synthesis of the (benzimidazolyl)isoquinolinol compound class, however the fluorescent conjugates were not successful as A1AR fluorescent ligands.

Introduction

Adenosine receptors (ARs) are class A G protein-coupled receptors (GPCRs), classified in four distinct subtypes - A1, A2A, A2B and A3. Adenosine A1 receptor (A1AR) is expressed throughout the human body with predominant distribution in brain, heart, kidney and adipose tissue and is an attractive therapeutic target. To this end, A1AR ligands are of interest and are being developed to treat various pathological conditions such as atrial fibrillation, angina pectoris, congestive heart failure, diabetes, neuropathic pain and renal disorders. Fluorescent ligands have emerged as useful and powerful tools to study real-time and live cell dynamic processes of GPCRs. Fluorescent ligands have been developed for ARs, including some with high AR subtype selectivity for A1AR. There is one report of fluorescent dansyl-linked N6 NECA derivatives with subtype selectivity for rat A1AR, however the short excitation wavelength of the dansyl fluorophore is not ideal for many applications. When the same research group conjugated the lead pharmacophore-linker to longer excitation wavelength fluorophores A1AR selective probes ensued. Therefore there remains a need to develop fluorescent ligands with high subtype selectivity for A1AR.

One approach to the design of a fluorescent ligand is to identify an existing pharmacophore with the requisite selectivity and affinity for the target receptor, and then identify a location that a linker and fluorophore can be attached. With this design in mind, it is then necessary to synthesise and test a range of pharmacophore, pharmacophore-linker and fluorescent ligands as modifications have the potential to alter the affinity and efficacy of the parent pharmacophore. In an effort to develop a selective A1AR fluorescent ligand we chose to avoid a xanthine amine congener (XAC)-based pharmacophore because of subtype selectivity concerns as a range of XAC-fluorescent conjugates have been reported as either non AR subtype selective or A2AR selective. Cosimelli et al. recently reported a series of (benzimidazolyl)isoquinolinols as selective antagonists for human A1AR (hA1AR), among them ligands 1–3 (Fig. 1), which were used as the inspiration for the fluorescent ligand design reported herein.

Structure–activity relationships (SAR) from the Cosimelli et al. study indicated small, lipophilic benzimidazole substituents such as methyl and ethylthio groups in some positions improved A1AR affinity and in others abolished it. This sensitivity to slight positional change, along with potential buried, lipophilic interactions these groups could be making with the A1AR to enhance affinity led us to discount linker substitution from the benzimidazole ring. Since 1,3-substituted isoquinoline analogues such as VUF5455 (ref. 9) and LUF6096 (ref. 10) have...
been reported as allosteric ligands for A1AR, albeit with quite different substituents, we chose to instead explore the previously unexplored isoquinoline C5–8 positions for linker attachment. A1AR ligands such as 1–3 lack a reactive functional group for linker and subsequently fluorophore attachment in these positions, therefore we designed a series of (benzimidazolyl)isoquinolines. To the best of our knowledge, there are no previous literature reports on the synthesis of (benzimidazolyl)isoquinolines. The hydroxyl group offers accessible chemistry for linker attachment, e.g. via an ether bond that remains unionised at physiological pH. Succinimidyl-6-[2-((E)-2-[4,4-difluoro-5-(2-thienyl)-3a,4a-diaza-4-bora-5-endo]-ethenyl)phenox]-acetylamino]hexanoate (BODIPY630/650-SE) was chosen as the fluorophore for coupling because of its success in developing fluorophores for studying GPCRs in previous studies and its suitable spectroscopic properties as an acceptor in bioluminescence resonance energy transfer (BRET) assays.

We envisaged using the Pictet–Spengler reaction in our synthesis to form a hydroxytetrahydroisoquinoline carboxylic acid followed by aromatisation to give a hydroxyisoquinoline carboxylic acid. This required an aromatisation reaction tolerant of functional groups such as a phenol, amine and carboxylic acid. Although isoquinoline is an important pharmacophore in various natural products, metal co-ordination ligands and marketed drugs such as papaverine, ripasudil, quinviscaine and moxaverine, a general method for the construction of isoquinolines with broad functional group tolerance, including the synthesis of isoquinolines, is lacking. Aromatisation of tetrahydroisoquinoline to isoquinoline is usually carried out using heavy metals such as palladium with harsh conditions with poor functional group tolerance.

Protection/deprotection of functional groups is another common strategy employed for successful aromatisation to prepare functionalised isoquinolines. Use of 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) has been reported for the aromatisation of tetrahydroisoquinoline and dihydroisoquinoline to isoquinolines at high temperature. In this study, we explored the use of DDQ to aromatise tetrahydroisoquinolines to isoquinolines under mild conditions as an alternate to heavy metal-based or harsh reaction conditions.

**Results and discussion**

The synthesis of 1-substituted 6-hydroxy tetrahydroisoquinoline began with attempts to carry out a Pictet–Spengler reaction of 3-[2-aminoethyl]phenol hydrobromide with glyoxylic acid monohydrate following the procedure Li et al. (no spectroscopic data provided for 5, ref. 29), however these were unsuccessful. Pleasingly, following the synthesis of 5 reported by Maillard et al. and Maillard et al. procedures being addition of triethylamine to the amine hydrobromide salt prior to addition of glyoxylic acid monohydrate (Scheme 1). The neutralisation presumably meant the imine could form more readily and hence the Pictet–Spengler reaction could proceed. Attempts to aromatise carboxylic acid 5 using DDQ and isolate the hydroxyisoquinoline carboxylic acid (using optimised conditions described below) were problematic, in part due to difficulty analysing and purifying the polar carboxylic acid. Therefore 5 was esterified to give methyl ester 6 (compound 6 was previously reported by Ma et al. but with no spectroscopic data). Synthesis of the 3-substituted tetrahydroisoquinolinol series began with Pictet–Spengler condensation of (+/-)-m-tyrosine with formaldehyde to give 9 according to a literature procedure reporting the synthesis of 9, followed by esterification to give 10. The synthesis of methyl ester 10 from 9 has previously been reported however none of these reports include spectroscopic data for 10. The first attempt to synthesise the 7-hydroxyl tetrahydroisoquinoline series via condensation of tyramine with glyoxylic acid monohydrate according to the procedure for 5 (except without trimethylamine as the amine was not a salt) was not successful. Failure of this reaction was attributed to the lack of an activating group in tyramine, compared to the presence of an activating para hydroxyl relative to the point of condensation for 4 and 8. Instead, Fmoc-protected tyramine was reacted with glyoxylic acid monohydrate to give a carboxylic acid according to the synthesis of this carboxylic acid reported by Maillard et al. which was esterified to give 13 in low yield over 2 steps. NMR spectra of 13 showed a mixture of isomers that were confirmed as rotamers rather than regioisomers via variable temperature NMR experiments.

There is one report of the aromatisation of an unprotected hydroxytetrahydroisoquinoline (10) with DDQ in patent literature (conversion of 10 to 11, no spectroscopic data for 11 provided) however in our hands all initial attempts to aromatise 6 or 10 using DDQ with varying temperatures and solvents and with either oxygen excluded or in a closed reaction vessel failed. When 6 and DDQ were dissolved in THF and dioxane and heated to 100 °C under a N₂ atmosphere the partially oxidised methyl 6-hydroxy-3,4-dihydroisoquinoline-1-carboxylate was isolated as the major product. However, at moderate temperature and with vigorous stirring in a flask open to the atmosphere, 6 and DDQ were reacted to give the desired product 7 in moderate yield. To our knowledge,
isoquinoline 7 has only been reported once before in the literature in a Japanese patent but no spectroscopic data was provided. The procedure to convert 6 to 7 was also used to successfully aromatise 10 to 11. Since the aromatised product did not form without vigorous stirring open to the atmosphere it is likely that DDQ mediates formation of a dihydroisoquinoline, which is then aromatised by oxygen as the active oxidant. Oxygen has previously been reported as an oxidant in aromatisation reactions and indeed Dong et al. used a base in dimethyl sulfoxide (DMSO) along with oxygen from air for conversion of N-tosyltetrahydroisoquinolines into isoquinolines. Dong et al. also isolated an imine under an argon atmosphere instead of the isoquinoline product, analogous to the partially oxidised imine intermediate (methyl 6-hydroxy-3,4-dihydroisoquinoline-1-carboxylate) isolated in our study with exclusion of air. Formation of an iminium ion intermediate (3,4-dihydroisoquinoline) has also been reported for DDQ-based cross-dehydrogenative coupling reactions of tetrahydroisoquinolines.

Diethylamine was initially used for Fmoc deprotection of 13 however subsequent aromatisation with DDQ provided poor yield of 14, most likely due to residual diethylamine. High temperature NMR spectra in DMSO-\(\text{d}_6\), carried out to study rotamers of 13 revealed cleavage of Fmoc, and indeed there is precedent in the literature that Fmoc cleavage can be conducted in neat DMSO at high temperature. Thus, Fmoc cleavage of 13 in DMSO, followed by DDQ and air mediated aromatisation afforded 14 in comparable yield to 7 and 11. The moderate yield of DDQ-air mediated aromatisation is unclear but may be due to low solubility in organic solvent of these compounds and susceptibility to methyl ester hydrolysis during basic work up.

Hydrolysis of 7 and 14 under basic conditions provided 15 and 16, which were condensed with o-phenylenediamine or 2,4-diaminotoluene in hot polyphosphoric acid (PPA) to afford 1- (benzimidazolyl)isoquinolinolines 17–19 in low yield (Scheme 2). This low yield was likely due to a number of factors, including poor double dehydration yield and a difficult isolation of a product with low organic solvent solubility from the viscous reaction mixture. NMR spectra of 17–19 showed a mixture of conformers, which was elucidated to be tautomers using variable solvent and high temperature NMR experiments and high performance liquid chromatography (HPLC) (discussed in ES†).

The phenolic group of 17–19 was reacted with tert-butyl bromoacetate to afford corresponding tert-butyl esters 20–22, which were reacted with trifluoroacetic acid to give carboxylic acids 23–25. Carboxylic acid 24 was coupled to three different amino linkers – a \(\text{C}_6\) alkyl, short polyethylene glycol (PEG) or \(\text{L-}\) Ala-\(\text{L-}\) Ala short peptide to give 27, 28 or 29 respectively. Carboxylic acid 23 underwent amide coupling to give PEG-linked 26 while the 7-substituted carboxylic acid 25 was converted to PEG-linked 30. These linkers were selected to explore variations in the physicochemical properties of the resulting fluorescent probes, in particular in light of lipophilicity and non-specific membrane binding. Boc deprotection of 26–30 provided amines that were reacted with BODIPY630/650-SE to fluorescent conjugates 31–35. 3-(Benzimidazolyl)isoquinolinol-based fluorescent conjugates 42 and 43 were synthesised using analogous methodology (Scheme 3). Absorption and emission spectra of fluorescent compounds 31–35 and 42–43 showed excitation maxima at 624 nm and emission maxima at either 641 nm or 642 nm (detailed in ES†).

**Pharmacology**

Fluorescent ligands 31–35 and 42–43 were analysed for their ability to bind to \(\text{A}_1\)-AR according to a previously reported bioluminescence resonance energy transfer (NanoBRET) saturation binding assay using the novel luciferase NanoLuc (NLuc; Promega Corporation, USA). HEK293 cell lines stably transfected with N-terminal NLuc labelled \(\text{A}_1\)-AR were treated with increasing concentrations of the (benzimidazolyl)isoquinolinol fluorescent ligands, in the presence and absence of 1 \(\mu\)M DPCPX (a high affinity, non-fluorescent, selective \(\text{A}_1\)-AR antagonist) to determine levels of non-specific binding.

![Scheme 1](image-url)
Unfortunately, all ligands 31–35 and 42–43 did not show any indication of binding to A1AR, for example data shown for 31 and 33 (Fig. 2a and b) (other compound data in ESI Fig. S16†).

Fluorescent ligands 31–34 were then analysed for their ability to bind to NLuc-labelled A3AR in analogous NanoBRET experiments, using A3AR selective MRS1220 to establish non-specific

Scheme 2 Synthesis of 1-(1H-benzimidazol-2-yl)isoquinolinols. Reagents and conditions: (i) LiOH, THF, H2O, 70–96%; (ii) PPA, 250 °C, 21–38%; (iii) tert-butyl bromoacetate, K2CO3, THF, 60 °C, 66–96%; (iv) TFA, DCM, 60% – quantitative; (v) HATU, DIPEA, DMF, 29–69%; (vi) BODIPY630/650-SE, DIPEA, DMF, 33–84%.

Scheme 3 Synthesis of 3-(1H-benzimidazol-2-yl)isoquinolinols. Reagents and conditions: (i) LiOH, THF, H2O, 90%; (ii) PPA, 250 °C, 26%; (iii) tert-butyl bromoacetate, K2CO3, THF, 60 °C, 94%; (iv) TFA, DCM, quantitative; (v) HATU, DIPEA, DMF, 37–39%; (vi) BODIPY630/650-SE, DIPEA, DMF, 73–86%.
binding. Ligands 32 and 34 did not show any specific binding to NLuc-A3AR (data not shown), while 31 and to a lesser extent 33 (Fig. 2c and d) showed a small degree of binding to NLuc-A3AR. However, the specific binding window was small and the standard error’s of the calculated \( K_d \) values for specific binding to NLuc-A3AR were large (31 \( K_d = 162 \pm 65.5 \text{ nM, } n = 5 \); 33 \( K_d = 534 \pm 254 \text{ nM, } n = 4 \)).

This NanoBRET assay is an excellent way to assess new fluorescent ligands\(^{44}\) since it measures the interaction of the fluorescent ligand and receptor directly without relying on the ability of the test ligand to compete with another tracer such as a radioligand. In this study the level of fluorescent ligand non-specific/membrane-binding was defined by measuring BRET in the presence, for A3AR, of a competing high concentration of unlabelled MRS 1220. Therefore this measure of specificity is defined by the ability of MRS 1220 \((a[1,2,4]triazolo[1,5-c]quinazolinyl pharmacophore) to prevent binding of the fluorescent compounds, which implies each would be interacting with the same or an overlapping region of the ligand binding pocket of the receptor. It is clear from Fig. 2c and d, however, that the non-specific binding component (obtained in the presence of MRS 1220) is not linear and is better fit with a saturable binding curve. It therefore remains a possibility (along with non-specific membrane interactions) that 31 and 33 may be interacting with an additional region of A3AR distinct from the orthosteric binding site of MRS 1220.

A possible explanation for the lack of binding of fluorescent ligands 31–35 and 42–43 to NLuc A3AR, is steric occlusion of binding due to the presence of the N-terminal NanoLuc tag. To investigate this, NanoBRET was used to quantify the specific binding of CA200645, a known fluorescent non-selective AR ligand at NLuc A3AR. Saturable binding was observed, with minimal non-specific binding and a large observation window (Fig. 3A; \( K_d = 110 \text{ nM + 18.46 } n = 5 \)) consistent with previous NanoBRET observations of CA200645 binding to NLuc A3AR or NLuc A1AR.\(^{12}\) NanoBRET assay plates were then washed and imaged using a IX Ultra confocal plate reader (MetaXpress, Molecular Devices) with fluorescence intensity quantified per well using a modified multiple wavelength cell scoring algorithm.\(^{12}\) Saturable binding of CA200645 was observed at NLuc A1AR \((K_d = 189.3 \text{ nM } \pm 69.6; n = 4, \text{ data not shown})\), with a \( K_d \) value consistent with that measured by NanoBRET (Fig. 3A). The binding of CA200645 to untagged A1AR stably expressed in HEK293, was also quantified using this fluorescent imaging-based technique with the resultant estimated binding affinity comparable to that seen for NLuc tagged A1AR \((144.8 \text{ nM } \pm 63.6; n = 3, \text{ data not shown})\). From these results it can be concluded that the NanoLuc tag does not occlude binding of CA200645 to

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**Fig. 2** HEK293 cells stably transfected with N-terminally NLuc-tagged A1AR or A3AR were treated with increasing concentrations of fluorescent ligand and the BRET ratio measured after direct addition of the NLuc substrate furimazine (10 \( \mu \text{M}\)).\(^{12}\) Non-specific binding was assessed in the absence and presence of 1 \( \mu \text{M} \) DPCPX (NLuc-A1AR) or 1 \( \mu \text{M} \) MRS1220 (NLuc-A3AR). Pooled data of raw BRET ratios were baseline corrected (minus vehicle + furimazine BRET ratios) so that data are expressed as fold increase in BRET ratios over basal. (a) NLuc-A1AR and (b) NLuc-A3AR and the BRET ratio measured after direct addition of the NLuc substrate furimazine (10 \( \mu \text{M}\)).

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| Concentration (nM) | Compound 31 | + 1 \( \mu \text{M} \) DPCPX | Compound 33 | + 1 \( \mu \text{M} \) MRS1220 |
|-------------------|-------------|-----------------|-------------|-----------------|
| 0                 | -0.002      | 0               | -0.002      | 0               |
| 100               | -0.002      | -0.002          | -0.002      | -0.002          |
| 300               | -0.002      | -0.002          | -0.002      | -0.002          |
| 500               | -0.002      | -0.002          | -0.002      | -0.002          |
| 600               | -0.002      | -0.002          | -0.002      | -0.002          |

**NLuc A3AR**

(c) NLuc-A3AR and (d) NLuc-A1AR and 31 and (d) NLuc-A3AR and 33. Data represents four–seven independent experiments (in triplicate).
the receptor, therefore it seems unlikely this tag is the reason for
the poor binding of fluorescent ligands 31–35 and 42–43.

In light of the poor binding of fluorescent ligands 31–35 and
42–43 to A1AR as measured using NanoBRET competition
binding experiments, a subset of (benzimidazolyl)isoquinolinols
described in Schemes 2 and 3 (1, 17–19, 28, 30, 37 and 40)
and literature reference compound 1 were tested using
NanoBRET competition assays. HEK293 NLuc A1AR cells were
expressing hA1AR.

Baseline corrected (minus vehicle + furimazine BRET
ratios) so that data are expressed as fold increase in BRET
ratios over basal and where appropriate fit using one site
saturation binding (mean ± SEM). For competition experiments
(B), NLuc A1AR were co-incubated with a fixed concentration
of CA200645 and increasing concentrations of unlabelled ligand
(1 h at 37 °C). Total CA200645 binding and vehicle are shown
by the black and white bars respectively. Data were pooled from
five independent experiments and are expressed as mean ± SEM.

**Experimental**

Full details of the synthesis of 1, 4–43, including all
methodology, chemical synthesis, pharmacology, equipment and
compound characterisation can be found in the ESI,† along
with a comprehensive tautomer study of 18. All compounds
tested in the BRET assay were shown by analytical HPLC to
possess >95% purity. As a representative examples of a DDQ
aromatisation reaction, conversion of 6 to 7 was as follows:
to a solution of 6 (0.17 g, 0.83 mmol) in 1,4-dioxane : THF
(10 mL 1 : 1, v/v) at 45 °C was added DDQ (0.38 g, 1.67 mmol) and the
reaction was stirred vigorously at 45 °C for 5 h with the mouth
of the flask open to the atmosphere to allow mixing of air. 1,4-
Dioxane (10 mL) was added and the reaction mixture filtered,
the filtrate was diluted with EtOAc and washed three times with
sat. NaHCO3 solution. The organic washings were combined,
wareshoned with water, brine solution, then dried over MgSO4,
concentrated under reduced pressure and purified by silica gel
flash column chromatography (30–50% EtOAc/hexane) to
provide 7 (84 mg, 0.413 mmol, yield 49%) as an off-white solid.

1H NMR (400 MHz, MeOD-d4) δ 4.04 (s, 3H, OCH3), 7.16 (d, J =
2.5 Hz, 1H, ArH), 7.28 (dd, 1H, J = 2.5, 9.3 Hz, ArH), 7.73 (d, 1H, J =
5.7 Hz, ArH), 8.30 (d, 1H, J = 5.7 Hz, ArH), 8.52 (d, 1H, J =
9.3 Hz, ArH). 13C NMR (101 MHz, MeOD-d4) δ 53.24, 108.72,
122.70, 122.89, 124.25, 129.49, 141.07, 141.83, 149.28,
161.18, 167.61. HRMS calculated for C11H10NO3 (M + H)+,
204.0655; found, 204.0647.

**Conclusions**

Our overall goal was to design new A1AR selective fluorescent
ligands, however it can be concluded that a (benzimidazolyl)iso-
quinolinol scaffold linked via the C5 or C6 isoquinolinine position
to a fluorophore is not suitable for this purpose. Although explora-
tion of other regions of the (benzimidazolyl)isoquinoline scaffold
(such as from the benzimidazole) for linker attachment are
possible, in light of the poor A1AR binding observed for 1, we
the selection of a different A1AR selective pharmacophore may be
optimal. Although the end biological aim of A1AR fluorescent
ligands was not achieved, a method for aromatisation of tetracy-
hydroisoquinolinols to give isoquinolinols and conformational
(tautomer) studies of (benzimidazolyl)isoquinolinols are reported.

**Fig. 3** HEK293 cells stably expressing N-terminal NLuc tagged
A1AR were treated with increasing concentrations of CA200645
(5–500 nM; 1 h at 37 °C; A). Non specific binding was defined using
1 μM DPCPX, an A1AR selective antagonist. The NanoLuc substrate
furimazine was added (10 μM), with luminescence and fluorescence
emissions recorded using a PheraStar FS. Data was pooled from
independent experiments (n = 5) and baseline corrected (minus vehicle + furimazine BRET
ratios) so that data are expressed as fold increase in BRET
ratios over basal and where appropriate fit using one site
saturation binding (mean ± SEM). For competition experiments
(B), NLuc A1AR were co-incubated with a fixed concentration
of CA200645 and increasing concentrations of unlabelled ligand
(1 h at 37 °C). Total CA200645 binding and vehicle are shown
by the black and white bars respectively. Data were pooled from
five independent experiments and are expressed as mean ± SEM.
Since there are only two reports for non-metal-mediated aromatisation of unprotected tetrahydroisoquinolinol to isoquinolinol, for which experimental details are unclear and no yields are provided, the method described herein using DDQ and atmospheric oxygen in mild conditions will prove useful for the preparation of biologically important isoquinolines and isoquinolinols.

Conflicts of interest

There are no conflicts to declare.

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