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Discovery of benzothiazolylquinoline conjugates as novel human A_3 receptor antagonists: biological evaluations and molecular docking studies

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Adenosine is known as an endogenous purine nucleoside and it modulates a wide variety of physiological responses by interacting with adenosine receptors. Among the four adenosine receptor subtypes, the A_3 receptor is of major interest in this study as it is overexpressed in some cancer cell lines. Herein, we have highlighted the strategy of designing the hA_3 receptor targeted novel benzothiazolylquinoline scaffolds. The radioligand binding data of the reported compounds are rationalized with the molecular docking results. Compound **6a** showed best potency and selectivity at hA_3 among other adenosine receptors.

1. Introduction

Adenosine is an endogenous purine nucleoside which regulates many physiological functions through the activation of four specific receptor subtypes, classified as A_1, A_{2A}, A_{2B} and A_3 adenosine receptors (AR), belonging to the family of G-protein-coupled receptors [1]. These receptors are widely distributed in mammalian tissues. The A_3AR subtype is the most recently characterized member of the family which was first cloned from a rat testis cDNA library [2] and is still undergoing intensive pharmacological characterization. The A_3AR subtype is implicated in various pathological conditions such as cardiac and cerebral ischaemia, neurodegenerative diseases as well as inflammatory pathologies including rheumatoid arthritis and asthma [3]. Furthermore, A_3AR is overexpressed in various

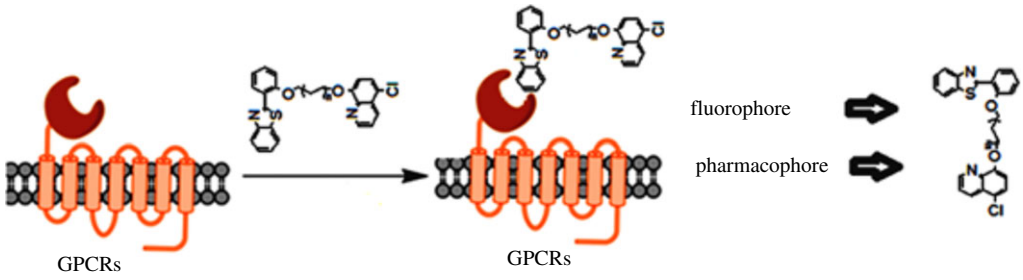


Figure 1. Dual role: pharmacophore as well as a fluorophore. G-PCR, G-protein coupled receptor.

neoplastic cells including HL-60 leukaemia, human Jurkat T-cell lymphoma, astrocytoma, A378 melanoma, B16-F10 and solid tumour (e.g. a two–threefold increase in colon carcinomas), while low or almost no receptor expression was found in normal cells [4,5].

Similar results were found in studies of the receptor expression levels in tumour tissues derived from patients with colon, breast, small cell lung, pancreatic and hepatocellular carcinomas and melanoma in direct comparison with adjacent body normal tissues [6–15]. Higher A_3AR expression in the tumour versus adjacent non-neoplastic tissue was further confirmed by reverse transcription-PCR analysis of colon and breast carcinoma. Protein analysis of A_3AR expression in fresh tumours derived from colon ($n = 40$) or breast ($n = 17$) revealed 61% and 78% higher expression in the tumour than adjacent normal tissue, respectively [16]. Thus the A_3 receptor could be a prospective therapeutic target and biological predictive marker in cancer therapy. The high A_3AR expression level in the tumour tissues was associated with elevated nuclear factor κB and cyclin D1 levels [16]. High A_3AR mRNA expression was also exhibited in other solid tumour types. Mechanistic studies demonstrated that A_3AR activation by synthetic agonists or antagonists induces down-regulation of key cell growth-regulatory proteins including cyclin D1 and nuclear factor κB [10,17,18]. Hence, discovery of selective A_3AR targeting ligands has been a great challenge in last two decades. Moreover, A_3AR antagonists research not only aids in the development of therapeutic agents but also in the development of diagnostic agents [2,19]. Nowadays, the diagnostic approaches have been significantly developed by using fluorescently labelled pharmacophores.

In the past few years, there have been strenuous efforts to develop different heterocyclic scaffolds as hA$_3$antagonists including pyridine and dihydropyridine analogues, flavonoid, isoquinoline, triazoloquinazolines, pyrazolo-[3,4-*c*]or-[4,3-*c*]quinolones, triazoloquinoxaline, pyrazolo-[4,3-*e*]1,2,4-triazolo-[1,5-c]pyrimidines, ruthenium-pyrazolopyrimidines [20–29]. However, none of the pharmacophores has been tested in A_3 overexpressed cancer cell lines. The cellular imaging study and cell surface receptor localization study was also not performed because these scaffolds are nonfluorescent. Therefore, to identify the hA$_3$ targeting novel fluorescent ligands, a molecular simplification followed by molecular docking approach was employed.

Benzothiazole possesses several biological activities such as anti-inflammatory, antimicrobial, anti-HIV, anticancer and amyloid marker [30–36]. These scaffolds are also able to arrest metal promoted amyloid fibril build-up [37]. Likewise, 8-hydroxy quinoline has also been developed as potent bioactive scaffold [38]. Herein, we have highlighted the strategy of designing the novel 2-(2′-hydroxyphenyl)benzothiazole (HBT) scaffolds having dual properties (pharmacophore and fluorophore) via molecular simplification followed by a molecular docking approach (figure 1). The designed molecules have already shown potency and selectivity in hA$_3$AR overexpressed cancer cell lines than normal cell lines [38]. In our earlier report, the cellular localization was also observed using those scaffolds. To justify the molecular pathway of these drugs, we have initiated the molecular docking approach as well as radioligand binding study at hA$_3$AR.

2. Results and discussion

2.1. Chemistry

2.1.1. Molecular simplification

Following a molecular simplification approach, we have identified the benzothiazolylquinoline ring system as an appropriate core skeleton for the design of novel fluorescent hA$_3$AR antagonists. Here,

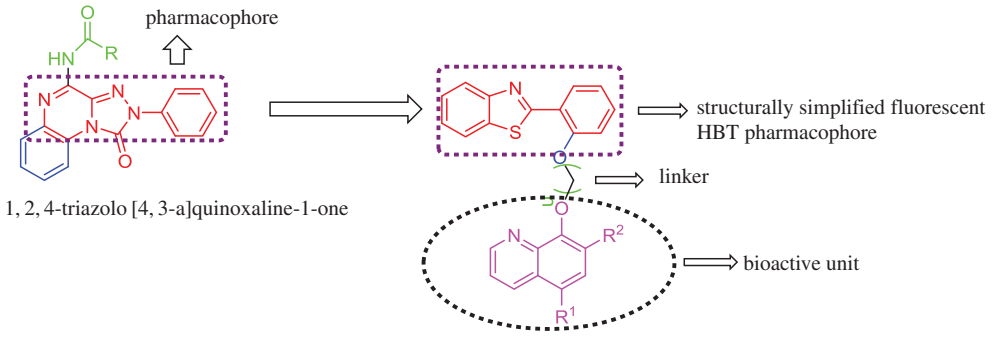
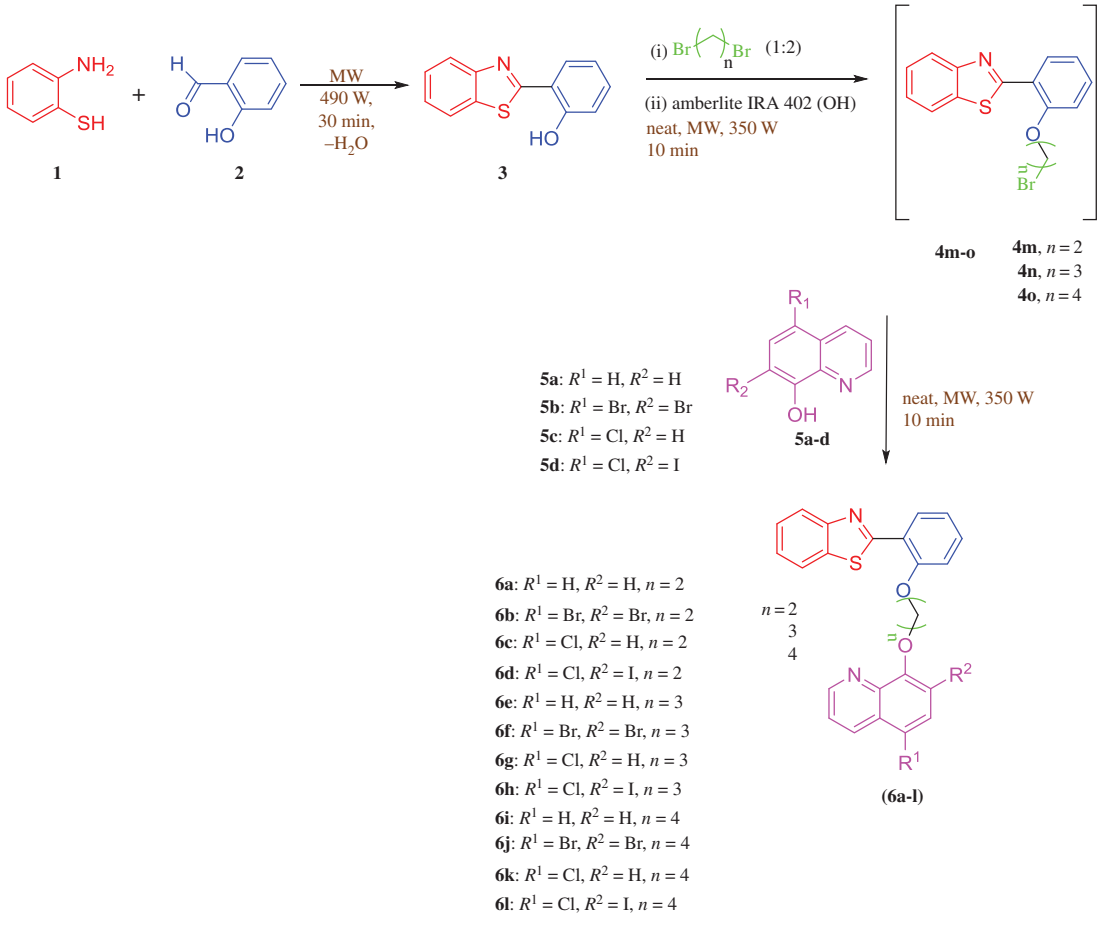


Figure 2. Molecular simplification approach to design the *h*A_3 targeting ligand.



Scheme 1. Preparation of 8-[2-(2-benzothiazol-2-yl-phenoxy)-alkoxy]-quinoline derivatives (**6a-l**) [38].

we have simplified the structure of the tricyclic A_3 antagonist (triazoloquinoxaline) into bicyclic motif (2-benzothiazolyl phenol). In the course of structure design, we have attached the bicyclic fluorescent pharmacophore (2-benzothiazolyl phenol) with another bioactive 8-hydroxy quinolone unit through a linker to enhance the A_3 binding ability (figure 2).

2.2. Synthesis

In our earlier report, a series of 2-(2′-hydroxyphenyl)benzothiazolylquinoline scaffolds were prepared in conventional way and also under microwave condition in one pot sequence (scheme 1) [38]. We followed the earlier reported green method to synthesize the following scaffolds and then characterized

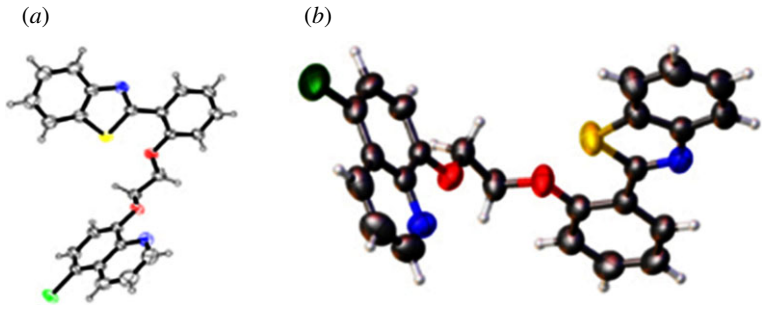


Figure 3. ORTEP diagrams of compound **6c** drawn at the 50% probability level [38].

Table 1. Binding energy of benzothiazolylquinoline analogues (**6a-l**) with hA_3.

entry	compound	GLIDE score (Kcal mol^{-1})
1	**6a**	−10.31
2	**6b**	−9.06
3	**6c**	−6.50
4	**6d**	−8.90
5	**6e**	−9.05
6	**6f**	−8.81
7	**6g**	−9.04
8	**6h**	−8.78
9	**6i**	−7.48
10	**6j**	−8.37
11	**6k**	−9.05
12	**6l**	−9.36

by ^{1}H NMR, ^{13}C NMR and LCMS study (electronic supplementary material, supporting information). Structure of compound **6c** was further confirmed by single crystal X-ray study (figure 3) [38].

2.3. Molecular docking studies

2.3.1. Homology modelling

The crystallographic structure of $hA_{2A}AR$ complexed with ZM-241385 as a high affinity antagonist (PDB code: 3EML) [39]. It was already being used to build up a homology model of the hA_3AR by our group [40]. Considering the high resolution (2.6 Å) and accuracy of the structure of $hA_{2A}AR$, 3EML was selected as a template by various research groups [41,42]. MODELLER 9.11 was used to perform the homology modelling [43–46] and the quality of the model was evaluated using the Ramachandran plot. Subsequently, the prediction ability of the constructed hA_3AR homology model was evaluated in the molecular docking experiments using the GLIDE tool from Schrödinger maestro.

2.3.2. Molecular docking

Our approach was to select a new class of hA_3AR targeting compounds from a group of hypothetically designed 2-phenylbenzothiazole (HBT)-based scaffolds. We performed molecular docking of 12 different HBT-based ligands using the GLIDE tool from Schrödinger maestro to identify the hypothetical binding mode at the hA_3AR. Finally, we have identified the best inhibitor for targeted hA_3AR from GLIDE scores (table 1).

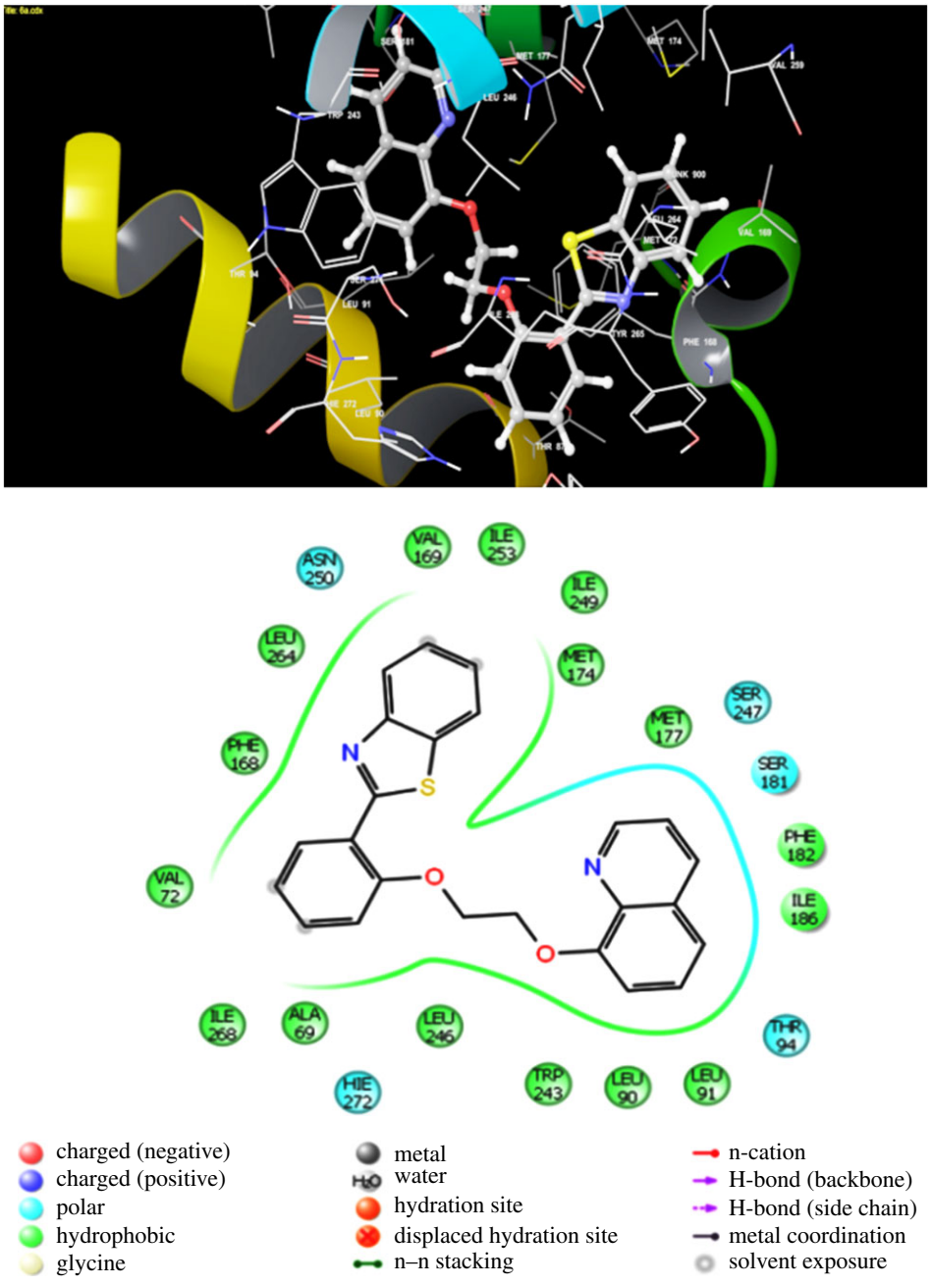


Figure 4. Binding interaction of most potent benzothiazolylquinoline analogue (**6a**) with hA_3 receptor.

All the newly synthesized benzothiazolylquinoline scaffolds were docked into the orthosteric transmembrane-binding cavities of hA_3AR. From the ligand docking, we have inferred that out of 12 synthesized scaffolds, compound **6a** displayed the best GLIDE score with the lowest binding energy. In figure 4, the hypothetical binding pose of compound **6a** is clearly observed at the hA_3AR. In particular, the most prominent aromatic π–π stacking interactions are established between Phe 168 and ligands and it was anchored properly within the binding cleft. Moreover, a strong hydrogen bond with Phe 168 was also appeared within the binding pocket. Likewise, a strong π–π stacking interaction was observed between Tyr 265 and compound **6f**. Compound **6g** also formed a hydrogen bond with Val 169 (electronic supplementary material, figure S1)

2.3.3. Binding affinity at hA_1AR, hA_{2A}AR, hA_{2B}AR and hA_3AR

The receptor binding affinities of the synthesized benzothiazolylquinoline derivatives (**6a-l**) are recapitulated in table 2. The binding affinity of the antagonist was estimated by measuring the

Table 2. Binding affinity (K_i) of synthesized compounds at hA_1AR, hA_{2A}AR and hA_3AR and selectivity against hA_1AR and hA_{2A}AR.

	K_i, μM (95% CI)[d]				selectivity	
compound	hA_1[b]	hA_{2A}[c]	hA_{2B}[a]	hA_3[e]	hA_1/A_3	hA_{2A}/A_3
6a	>100	23.8 (21.6–26.4)	>30	2.6 (1.8–4.5)	>38.46	>11.53
6b	>100	17.2 (15.3–19.4)	>20	3.2 (2.4–4.1)	>31.25	>6.25
6c	30.8 (26.5–32.7)	32.8 (28.7–34.1)	>20	30.4 (27.6–32.1)	1.01	>19.80
6d	>100	>20	>20	5.6 (3.8–7.1)	>17.85	>3.57
6e	>100	>20	>30	3.8 (2.6–4.7)	>26.31	>7.89
6f	>100	>20	>30	6.2 (4.8–7.1)	>16.12	>4.83
6g	>100	>20	>20	4.2 (3.1–5.4)	>23.80	>4.76
6h	>100	>20	>20	6.1 (4.9–7.2)	>16.39	>3.27
6i	29.7 (27.8–32.6)	34.9 (32.7–37.1)	>20	25.4 (23.6–27.1)	1.16	>0.78
6j	>100	>20	>20	6.4 (4.9–8.2)	>15.62	>3.15
6k	>100	>20	>10	3.7 (2.7–4.8)	>27.02	>2.70
6l	>100	>20	>30	3.6 (2.1–4.8)	>27.77	>8.33

[a]Adenylyl cyclase activity of synthesized compounds at the hA_{2B}AR.
[b]Displacement of specific [3H]-CCPA binding at hA_1AR expressed in Chinese hamster ovary (CHO) cells ($n = 3$–6).
[c]Displacement of specific [3H]-5′-*N*-ethylcarboxamido adenosine (NECA) binding at hA_{2A}AR expressed in CHO cells ($n = 3$–6).
[d]K_i values for inhibition of NECA-stimulated adenylyl cyclase activity in CHO cells ($n = 3$–6).
[e]Displacement of specific [3H]-2-(1-hexynyl)-N^6-methyl adenosine (HEMADO) binding at hA_3AR expressed in CHO cells ($n = 3$–6).

displacement of selective radioligands which were formerly bound to the receptor expressed (Chinese hamster ovary cells (CHO) for hA_1AR, hA_{2A}AR and hA_3AR) at the surface of the cell. In this particular assay, the displacement of: (i) specific [3H] CCPA (2-chloro-*N*(6)-cyclopentyladenosine) binding at hA_1AR, (ii) specific [3H] NECA (5′-*N*-ethylcarboxamidoadenosine) binding at the hA_{2A}AR, and (iii) [3H] HEMADO (2-(1-hexynyl)-N^6-methyladenosine) at the hA_3AR were evaluated. There is no suitable radioligand for hA_{2B}AR found and hence the antagonists activity was determined in adenylyl cyclase experiments in CHO cells expressing the hA_{2B}AR [47,48]. K_i (dissociation constant) value of the data was calculated using the Cheng and Prusoff equation [49], with geometric means of at least three experiments including 95% confidence intervals. From table 2, it was observed that most of the compounds exhibited a K_i value at hA_3 in the range of 2–6 μM. Compound **6c** and **6i** showed least binding efficacy with hA_3 which is clearly rationalized with their binding energy profile (GLIDE score). Compound **6a** exhibited the most binding potency at hA_3 in the 2.6 μM range. This compound has also shown 38-fold and 11-fold more selective potency at hA_3 than hA_1 and hA_{2A}, respectively. While increasing the length of the –CH_2– linker from 2 to 3, binding potency of the compound **6e** at hA_3 is reduced to 3.8 μM. Selectivity of this compound at hA_3 with respect to hA1 and hA_2 has also been reduced to some extent. Consequently, compound **6i** showed much less potency and selectivity in hA_3. Compounds (**6f-h** and **6j-l**) having electronegative groups and lengthy linkers ($n = 3$, 4) showed good potency and selectivity at hA_3. It was also observed that compound **6b** having two electronegative bromine groups with a small linker ($n = 2$) exhibited good potency (3.2 μM) and selectivity at hA_3.

3. Experimental

3.1. Biology

3.1.1. Chinese hamster ovary membranes preparation

The human A_1, A_{2A}, A_{2B}, and A_3ARs were transfected in CHO cells based on the previously reported method [47–50]. The cells were grown adherently and maintained in Dulbecco's modified Eagle's medium with nutrient mixture F12 (DMEM/F12) without nucleosides, containing 10% foetal calf serum, streptomycin (100 μg ml^{-1}), penicillin (100 μg ml^{-1}), L-glutamine (2 mM) and Geneticin (G418, 0.2 mg ml^{-1}) at 37°C in 5% CO_2, 95% air. The membrane preparation was initiated by removal of

culture medium followed by washing of the cells with phosphate buffered saline and scraped off T75 flasks in ice-cold hypotonic buffer (5 mM Tris–HCl, 2 mM EDTA, pH7.4). Then the cell suspension was homogenized with a polytron, and the homogenate was centrifuged for 10 min at 1000 g. The supernatant was then centrifuged for 30 min at 100 000 g. The membrane pellet was suspended in (i) 50 mM Tris–HCl buffer, pH 7.4, for A_1ARs; (ii) 50 mM Tris–HCl, 10 mM $MgCl_2$ buffer, pH 7.4, for A_{2A}ARs; and (iii) 50 mM Tris–HCl, 10 mM $MgCl_2$, 1 mM EDTA buffer, pH 7.4, for A_3ARs. The cell suspension was incubated with adenosine deaminase for 30 min at 37°C. Then the membrane preparation was used for binding experiments.

3.1.2. Binding at human A_1, A_{2A} and A_3 adenosine receptors

For radioligand binding at A_1 ARs 1 nM [3H] CCPA was used, whereas 30 nM of [3H] NECA were used for A_{2A} and 10 nM of [3H]- HEMADO were used for A_3 receptors, respectively [47–50]. Nonspecific binding of [3H]CCPA was determined in the presence of 1 mM theophylline, when [3H]NECA 100 µM R-PIA was used.

3.1.3. Adenylyl cyclase activity

The potency of antagonists at the A_{2B}AR was determined in adenylyl cyclase experiments [47–50]. For the measurement of adenylyl cyclase activity, only one high speed centrifugation of the homogenate was used. The resulting crude membrane pellet was resuspended in 50 mM Tris–HCl, pH 7.4 and immediately used for the cyclase assay.

3.1.4. Data analysis

Inhibitory binding constants, K_i, were calculated from the IC_{50} values according to the Cheng and Prusoff equation $K_i = IC_{50}/(1 + [C^*]/KD^*)$, where [C*] is the concentration of the radioligand and KD* its dissociation constant [49,50]. A weighted nonlinear least-squares curve fitting program LIGAND was also used for computer analysis of inhibition experiments. Potency values (IC_{50}) obtained in cyclic AMP assays were calculated by nonlinear regression analysis using the equation for a sigmoid concentration–response curve (Graph Pad Prism, San Diego, CA, USA). All experimental data are expressed as geometric mean with 95% confidence limits in parentheses of three or four independent experiments performed in duplicate.

4. Conclusion

In summary, we have designed a class of benzothiazolylquinoline scaffolds for the hA_3 target. Molecular simplification and molecular docking approach using the GLIDE tool from Schrödinger maestro has been employed for the design of these drugs. The effective binding modes of the scaffolds with the receptor binding sites were clearly explained. In addition, we have also performed radioligand binding assay of these scaffolds at hA_1AR, hA_{2A}AR, hA_{2B}AR and hA_3AR. We observed that compound **6a** exhibited maximum potency and selectivity in hA_3AR with respect to hA_1AR, hA_{2A}AR and hA_{2B}AR which is rationalized with a docking study. Finally, it was concluded that these cytotoxic molecules are selectively targeting to the hA_3AR.

Ethics. The department was ethically approved by UGC.

Data accessibility. Molecular docking images of selected compounds can be found in the electronic supplementary material.

Authors' contributions. B.S. and S.M. synthesized and characterized the compounds. B.S. has performed the molecular docking study. B.S. and S.M. contributed equally in this paper. G.R.J. did the radioligand binding assay for this paper. B.S., S.M. and G.R.J. drafted the manuscript. All the authors analysed and discussed the results and revised the manuscript.

Competing interests. We have no competing interests.

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