Design, synthesis, and biological evaluation of triazole-pyrimidine-methylbenzonitrile derivatives as dual A2A/A2B adenosine receptor antagonists

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A series of novel dual A2A/A2B AR antagonists based on the triazole-pyrimidine-methylbenzonitrile core were designed and synthesised. The A2A AR antagonist cAMP functional assay results were encouraging for most target compounds containing quinoline or its open-ring bioisosteres. In addition, compound 7I displayed better inhibitory activity on A2B AR (IC50 14.12 nM) and higher potency in IL-2 production than AB928. Moreover, molecular docking studies were carried out to explain the rationality of molecular design and the activity of compound 7I. Further studies on 7I and 7I revealed good liver microsomes stabilities and acceptable in vivo PK profiles. This study provides insight into the future development of dual A2A/A2B AR antagonists for cancer immunotherapy.

1. Introduction

Adenosine is one of the most important signalling molecules in the human body, and it exerts its effects through G-protein coupled receptors, including A1, A2A, A2B, and A3 adenosine receptors (ARs). Upon activation by adenosine, A2A AR and A2B AR promote adenylyl cyclase (AC) activation and subsequent cyclic AMP (cAMP) production. Elevated intracellular cAMP in T cells will result in T cell anergy by reducing its proliferation, maturation, cytokine production (e.g., IL-2), and tumour-killing activity. The cell cytotoxicities of natural killer cells, dendritic cells, or macrophages are inhibited by this pathway as well. In the tumour microenvironment (TME), the level of extracellular adenosine is higher than that of normal tissue, leading to immune evasion. A2A and A2B ARs are widely considered critical to the immune functions of adenosine. The relevance of A2 receptors in tumour immunotherapy has stimulated the development of various selective antagonists for these receptors.

In recent years, the discovery and translation of AR antagonist compounds to the bench for bedside for cancer immunotherapy have made significant progress, with some selective AR antagonists entering clinical trials either alone or in combination with other immunotherapies. Previous studies mostly focussed on the discovery of inhibitors against the A2A receptor. ZM241385 (Figure 1) is a very potent and selective A2A AR antagonist developed by the AstraZeneca Group. It can inhibit and delay tumour growth significantly. The structural analysis of A2A AR bound to ZM241385 confirmed the π–π stacking interaction between Phe168 and hydrogen bonds (H bonds) with Asn253 established by inhibitors were beneficial to improving the binding ability to the A2A receptor. The finding has facilitated the discovery of novel AR antagonists. For example, the A2A AR inhibitor CPI-444 (Figure 1) developed by Corvus Pharmaceuticals is considered to have a similar target binding form to ZM241385 and it is currently in a phase 1b/2 trial for the treatment of renal cell cancer.

Since the A2B receptor is also important in adenosine signal transduction, especially in cells of myeloid origin, a dual A2A/A2B AR antagonist usually exhibits better inhibition effects. Azolopyrimidines (e.g., compound A in Figure 2) were first disclosed by Arcus Biosciences as A2B AR antagonists. However, AB928 contains methylbenzonitrile, instead of the furan fragment of compound A, and it can inhibit A2A AR and A2B AR with similar potencies and outperform others in preclinical testing. In 2019, Selvita Group published a patent on imidazo[1,2-a]pyrazines for the treatment of tumour-related disorders. Among these imidazo[1,2-alpyrazines, SEL330-639 containing the quinoline structure was confirmed to be a dual A2A/A2B receptor antagonist with nanomolar potency. Through the structure-activity relationship of the above two compounds, methylbenzonitrile and quinoline structures may be advantageous scaffolds to obtain potent dual A2A/A2B AR antagonists. Incyte Corporation developed two types of A2A/A2B inhibitors (Figure 2, compounds B and C). Compounds containing benzonitrile structure or quinoline analogues also showed good inhibitory activities on both of these two receptors. Thus, we introduced quinoline or its open-ring bioisosteres to the structure of AB928 to develop new dual A2A/A2B AR antagonists. Herein, a series of novel A2 AR antagonists containing triazole-pyrimidine-methylbenzonitrile core were designed and synthesised (Figure 2, compounds D). Their potential inhibitory activities to A2A or A2B ARs were further investigated by cAMP functional assay and T cell activation assay. In addition, the representative compound 7I was subjected to molecular docking studies to reveal the binding behaviour. Moreover,
microsomal metabolic stability and in vivo pharmacokinetic properties were assessed for optimised compounds.

2. Results and discussion

2.1. Chemistry

The synthetic route of target compounds 7a–7l is shown in Scheme 1. Compound 2 was synthesised from the reaction of 3-bromo-2-methylphenylacetonitrile (1) with (BPin)$_2$ catalysed by Pd(dppf)Cl$_2$. Then, compound 3 was acquired from the Suzuki coupling reaction between compound 2 and 4,6-dichloro-2-pyrimidinamine. Subsequently, compound 4 was synthesised via the Sonogashira cross-coupling reaction of compound 3 with trimethylsilylacetylene (TMSA), which further reacted with tetrabutylammonium fluoride (TBAF) in THF to get compound 5. A copper and sodium L-ascorbate catalysed azide-alkyne coupling between 5 and 6 yielded compounds 7a–7b and 7d–7g. In addition, compound 7b could be reduced with SnCl$_2$ to obtain compound 7c. Furthermore, treatment of compound 7d with LiOH aqueous solution or various amine

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Figure 1. Chemical structures of A$_2$ adenosine receptor antagonists.

Figure 2. Design of dual A$_2A$/A$_2B$ AR antagonists containing quinoline or its open-ring bioisosteres based on the structure of triazole-pyrimidine-methylbenzonitrile.
derivatives yielded 7h–7l. The synthesis of intermediates 6 can be achieved by nucleophilic substitution of the corresponding alcohols or bromides with azidotrimethylsilane (TMSiA) or diphenyl azidophosphate (DPPA) with good yields (the synthesis of intermediates 6 can be found in the Supporting information).

To verify the effect of the benzonitrile and quinoline structure on A2 receptors, compounds 7m, 7n, 17, and 22 were synthesised following the synthetic routes shown in Scheme 1–3. Compounds 7m–7n, which contain a benzene ring instead of the quinoline structure, were synthesised by the azide-alkyne coupling between 5 and 6m–6n in 78–82% yield. The synthesis of compound 17 (Scheme 2) started from 5-methylfuran-2-boronic acid pinacol ester (11), according to the synthesis method of compound 7a, and 17 was generated in moderate yield. In the synthetic route of compound 22, the intermediate 21 synthesised from compound 18 can be purchased from Sundia Company (the synthesis of intermediate 21 is found in the Supporting information). Intermediate 21 then reacted with TBAF and intermediate 16 in the presence of CuSO4·5H2O and sodium i-ascorbate to obtain target compound 22 in a one-pot reaction.

2.2. Biology studies

2.2.1. cAMP functional assay
In the hypoxic TME, over-activation of A2 receptors upregulates CAMP levels by stimulating adenylate cyclase. The abilities of synthesised compounds 7, 17, and 22 to inhibit the hA2A or hA2B receptor were studied by evaluating their effect on cAMP production in Chinese hamster ovary (CHO) cells which stably express hA2A AR or hA2B AR4,9,37. The cAMP assay results are summarised in Table 1. In the cAMP assay, CHO-K1/ADORA2A/Ga15 cells expressing human A2A AR and CHO-K1/ADORA2b/Ga15 cells expressing human A2B AR were activated by 50-(N-ethylcarboxamido)adenosine (NECA) and triggered the accumulation of intracellular cAMP concentration. The agonist-induced cAMP intracellular accumulation was inhibited by compounds 7 or ZM241385. The inhibition rates of compounds 7 were determined by comparing the inhibitory activities of the target compounds at a certain concentration with that of ZM241385 at 1μM. From the cAMP assay results, compounds 7 exhibited good to excellent inhibitory activities on the NECA-triggered cAMP intracellular accumulation in CHO cells expressing human A2A AR (from 43.54% to 107.32%) at 1μM. It was noted that 7g and 7i showed superior inhibition activity to AB928, as a control. At 100 nM, compound 7a containing quinoline structure showed 16.52% and 3.51% inhibition, respectively, on A2A AR- and A2B AR-mediated CAMP production. It is worth mentioning that the introduction of an amino group as a hydrogen bond donor at C8 of the quinoline moiety was beneficial to the inhibitory activities (Table 1, 7a versus 7c). In particular, the inhibition of A2B AR-mediated cAMP production was increased to an impressive 85.43%. Moreover, the replacement of the phenyl ring of the quinoline structure by a ring-opening structure further increased the activity. Compounds 7d–7l, containing the pyridine structure, exhibited better inhibitory
Scheme 2. Synthetic route of the target compound 17. Reagents and conditions: (a) 4,6-dichloropyrimidin-2-amine, K₂CO₃, Pd(PPh₃)₄, DME, N₂, 45 °C, 5 min, then compound 11 was added, 115 °C, 5 h; (b) Et₃N, TMSA, Pd(PPh₃)₂Cl₂, CuI, dry THF, N₂, 10 °C, reflux, 16 h; (c) TBAF (1 M in THF), THF, 0 °C to r.t., 20 h; (d) MeMgBr, THF, r.t., 12 h; (e) DPPA, DBU, THF, r.t., 10 h; (f) CuSO₄·5H₂O, sodium L-ascorbate, 60 °C, DMF, t-BuOH, 12 h.

Scheme 3. Synthetic route of the target compound 22. Reagents and conditions: (a) MsCl, Et₃N, 0 °C, DCM, 1 h, then TBAF (1 M in THF) was added, 50 °C, 12 h; (b) 4,6-dichloropyrimidin-2-amine, TMSA, Et₃N, Pd(PPh₃)₂Cl₂, CuI, dry THF, N₂, 80 °C, 12 h; (c) Pd(dppf)Cl₂, K₂CO₃, 1,4-dioxane, N₂, 100 °C, 4 h; (d) TBAF (1 M in THF), CuSO₄·5H₂O, sodium L-ascorbate, H₂O, t-BuOH, 60 °C, 12 h.

Table 1. Antagonist activity (% inhibition) of compounds 7, 17, and 22 at 10–1000 nM on cAMP levels in CHO cells expressing hA2A AR or hA2B AR.

| Compound | Ar¹ | Ar² | hA2A AR (%) | hA2B AR (%) |
| --- | --- | --- | --- | --- |
| 1000 nM | 100 nM | 10 nM | 100 nM | 100 nM |
| 7a | | | 43.54 ± 0.77 | 16.52 ± 1.90 | 28.24 ± 8.79 | 3.51 ± 3.19 |
| 7c | | | 48.45 ± 1.45 | 38.16 ± 10.85 | 35.9 ± 5.39 | 85.43 ± 0.04 |
| 7d | | | 91.27 ± 4.28 | 63.38 ± 1.39 | 40.83 ± 9.01 | 80.27 ± 1.84 |
| 7e | | | 67.76 ± 4.91 | 50.68 ± 5.51 | 44.99 ± 1.25 | 82.45 ± 7.81 |
| 7f | | | 92.41 ± 0.43 | 84.21 ± 2.94 | 54.91 ± 1.19 | 102.64 ± 0.24 |
| 7g | | | 107.32 ± 0.89 | 56.02 ± 2.03 | 19.61 ± 2.13 | 52.94 ± 3.96 |
| 7h | | | 92.61 ± 1.91 | 55.18 ± 4.27 | 37.91 ± 2.32 | 54.75 ± 2.09 |
| 7i | | | 102.58 ± 2.82 | 95.93 ± 3.36 | 32.73 ± 1.80 | 103.33 ± 0.63 |
| 7j | | | 100.79 ± 4.62 | 82.17 ± 1.26 | 22.45 ± 6.68 | 82.38 ± 4.52 |

(continued)
activities against A2A AR than compound 7c (from 50.68% to 95.93%) at 100 nM, among them, 7f and 7i showed 84.21% and 95.93% inhibition, respectively, which were equivalent to that of AB928. Interestingly, at lower concentration (10 nM), they displayed higher inhibition rates than AB928. In addition, the inhibitory activities of 7f and 7i on A2B AR-mediated cAMP production were comparable to that of the control compound AB928.

The activities of compounds 7d–7l at 100 nM indicate the substituents at the pyridine structure could influence the inhibitory activities of these compounds on both A2 ARs. The substituent should be a group that contains a hydrogen bond receptor such as an ester donor such as –NH2 and –NH could also increase the inhibition activities of structures bearing carbonyl group (7d–7f versus 7i–7j) on both A2A AR and A2B AR. For compounds with an amide moiety, the size of the substituent at the nitrogen atom has a negative influence on the activity (7i > 7j > 7k). A hydrogen bond donor attached to the carbonyl group was necessary for the inhibitory activities of target compounds on A2A AR, but it has no obvious effect on the inhibitory activities on A2B AR (7d–7e, and 7f).

To further validate our design strategy, compounds 7m–7n, containing a benzene ring instead of the pyridine ring or quinoline structure, were synthesised. These compounds exhibited similar inhibitory activity to compounds 7a and 7c at concentrations of 1000 nM and 100 nM, but the activities were lower at 10 nM. Moreover, compounds 17 and 22 containing the same Ar1 fragment as AB928 were synthesised and showed good inhibitory activities at 10 nM, 100 nM, and 1000 nM against A2A AR. However, their activities against A2B AR decreased significantly at 100 nM because of the replacement of the Ar2 fragment by other structures. The changes in the activities of compounds 7m–7n, 17, and 22 relative to 7a, 7c–7l, and AB928 indicated the structures of benzonitrile and pyridine analogues are beneficial to improving the inhibitory activities of target compounds on human A2 receptor, which was consistent with our design strategy.

We further tested the IC50 values of compounds 7f, 7i, 17, 22, and AB928 in inhibiting the cAMP production of CHO cells expressing hA2A AR or hA2B AR (Table 2). The results indicate the inhibitory activity of compound 7i on the A2A receptor is similar to that of AB928, and compound 7f is 4-fold less active than compound 7i. The inhibitory activities of compounds 17 and 22 were 52-fold and 11-fold less active than compound 7i, respectively. In addition, the competitive binding experiments of compounds 7f, 7i, and AB928 were performed using membrane preparation of HEK-293 cells expressing human A2A AR (Table 3). Their IC50 values were 158.0 nM, 63.55 nM, and 25.23 nM, which are consistent with the results of the cAMP assay.

Compound 7i was also the most effective in inhibiting A2B AR-mediated cAMP production, and the IC50 value was 14.12 nM, which is 1.8-fold more active than compound AB928. Compounds without benzonitrile structure (17 and 22) had poor inhibitory activities indicating the cyano structure on the benzene ring is closely related to the inhibitory activity of the antagonist on A2B AR.

2.2.2. T Cell activation assay

The effector functions of T cells that express A2 ARs can be evaluated by measuring specific cytokine production6,9,38. In this study, the efficiencies of selected antagonists on IL-2 production (T cell activation) were examined. Two compounds (7f and 7i) were evaluated in this assay and showed sufficient activities to stimulate IL-2 production through T cell activation (Figure 3). The results show the concentration of IL-2 was significantly reduced in the NECA control, which suggests NECA inhibited T cell activation. However, when the antagonist was added, the concentration of IL-2 was restored, indicating the inhibition effect of NECA was blocked. Interestingly, 7i demonstrated better activity than AB928, suggesting it has potential as an immunotherapy against tumours via T cell activation.

2.2.3. Molecular docking studies

Docking studies were performed at the hA2A and hA2B AR binding sites to simulate the interaction of compound 7i with these two
Table 2. IC50 values of selected compounds on cAMP assays in CHO cells expressing hA2A or hA2B AR.

| Compound | cAMP IC50 (nM) |
|----------|----------------|
|          | hA2A AR | hA2B AR |
| 7f       | 24.04 ± 0.06 | 102.64 ± 0.24%
|          |          | 10.79 ± 0.67%
| 7i       | 6.007 ± 0.05 | 14.12 ± 2.05 |
| 17       | 320.5 ± 94.04 | 80.03 ± 13.45 |
| 22       | 67.19 ± 10.92 | 178.5 ± 9.89 |
| AB928    | 2.754 ± 0.12 | 25.48 ± 0.35 |

aData are expressed as means ± SEM.

bPercentage of inhibition (%) determined at 100 nM concentration of compound 7f.

cPercentage of inhibition (%) determined at 10 nM concentration of compound 7f.

Table 3. Radioligand binding affinity data (IC50) for selected compounds against hA2A AR.

| Compound | cAMP assays IC50 (nM) | Radioligand binding assays IC50 (nM) |
|----------|-----------------------|-------------------------------------|
| 7f       | 24.04 ± 0.06          | 158.0 ± 56.42                       |
| 7i       | 6.007 ± 0.05          | 63.55 ± 13.03                       |
| AB928    | 2.754 ± 0.12          | 25.23 ± 0.74                        |

aData are expressed as means ± SEM.

receptors. The binding modes of compound 7i at the hA2A AR cavity were analysed by docking simulations using the CDOCKER protocol of Discovery Studio 2017 R2. The crystal structure of hA2A AR in complex with antagonist ZM241385 (PDB ID: 5IU4) was retrieved from the Protein Data Bank (http://www.rcsb.org/pdb) for the docking calculations. The docking simulation results show that compound 7i could bind to the active site inside the transmembrane (TM) region and extracellular loops (ECLs) of the human A2A AR similar to the co-crystallized ZM241385. In this binding mode, the phenyl group is located in the proximity of His624, Leu167, Leu267, and Lys153 at the entrance of the hA2A AR cavity (Figure 4(a,c); for more details see Supporting information Figure S2). The pyridine and triazole scaffold position in the depth of the binding pocket and both give π-π interaction with the key receptor residue Phe168 (ECL2). This indicates the attachment of an aromatic ring to the triazole facilitates the binding of the inhibitor to the target. In addition, the asparagine residue in TM6 (Asn253) forms H-bond with the N1 atom at the pyridine scaffold and it forms an additional H-bond with the amide group attached to the pyridine ring. The docking results are consistent with the biological results.

The binding mode of hA2B AR was also analysed with the same docking protocols in Discovery Studio 2017 R2. The homology model of hA2B AR was developed using a hA2A AR crystal structure as the template (PDB ID: 6PS7) and has been checked using the Ramachandran plot application within Discovery Studio 40,41. In the binding mode (Figure 4(b,d); for more details see Supporting information Figure S3), the pyridine scaffold is located at the entrance of the cavity getting close to residue Phe173 in the ECL2 segment, and the methylbenzonitrile moiety is located in the depth of the binding pocket by a π-π interaction with Trp247. In addition, the cyano group has a polar interaction with the amide function of Asn282. The binding mode shows the methylbenzonitrile structure is beneficial to obtaining potent inhibitory activity on A2B AR. Moreover, favourable interactions with the amide group and methyl group are established with the side chains of Ala64 and His280. Thus, the substituents on the pyridine ring may affect the compound affinity.

2.2.4. Microsomal metabolic stability

The metabolic stability is an important focus for further compound optimisation. Compounds 7f, 7i, and AB928 were chosen to evaluate the stability in rat liver microsomes and human liver microsomes. For the microsomal metabolic experiment, 7-ethoxycoumarin (7-EC) was used as a control. As shown in Table 4 (see Supporting information for raw data), the CLint(liver) of 7-EC in rats and humans was 10.0 and 9.0 ml/min/g liver, respectively, indicating the experimental test system was reliable. Compounds 7f and 7i exhibited good liver microsomes stabilities in vitro. They hardly metabolised on human liver microsomes but showed slow metabolism on rat microsomes with CLint(liver) of 0.78 and 1.08 ml/min/g, and both compounds were more stable than AB928 (2.15 ml/min/g in rat liver microsomes).

2.2.5. In vivo pharmacokinetics study

The in vivo pharmacokinetic (PK) profiles of compounds 7f and 7i were examined in male Balb/c mice (Table 5 and Figure 5). The two compounds showed similar pharmacokinetic properties. After the intravenous (i.v.) administration of 10 mg/kg, the maximum plasma concentration of compounds 7f and 7i were 5010.30 μg/l at 0.25 h and 7266.60 μg/l at 0.25 h, respectively. When orally administered at 30 mg/kg, these compounds showed moderate half-lives at a range of 4.12–4.82 h. The absolute bioavailabilities of compounds 7f and 7i were 87.7% and 58.8%, respectively. These PK parameters can be used to evaluate the drug-like properties of compounds 7f and 7i, so as to clarify their effectiveness and obtain better clinical treatment effects.

3. Conclusion

A series of dual A2A/A2B AR antagonists containing structures of methylbenzonitrile and quinoline or pyridine analogues were designed and synthesised. In vitro cAMP assays of target compounds on A2A AR and A2B AR showed good to excellent inhibitory activities. Among these compounds, the inhibitory activity on the A2A receptor of compound 7i approached that of AB928. Additionally, compound 7i displayed better inhibitory activity on A2B AR and higher potency in IL-2 production than AB928. Further studies on 7i demonstrated good liver microsomes stabilities and acceptable in vivo PK properties. In future studies, we aim to further improve the potency and drug-like parameters of target compounds. Further optimizations of compound 7i are still in progress.

4. Experimental section

4.1. General methods for chemistry

All chemicals were purchased from commercial suppliers and used without further purification. Air or moisture sensitive reactions were performed under a positive pressure of nitrogen with oven-dried glassware. Reactions were monitored by thin layer chromatography (TLC), and spots were visualised with iodine vapour or by irradiation with UV light. Flash column chromatography was performed using the Qingdao Haiyang flash silica gel (200–300 mesh). All yields were reported as isolated yields. The melting points were determined on an X-4 binocular microscope melting point apparatus (Beijing Tech Instruments Co., Beijing, China) and were uncorrected. 1H NMR and 13C NMR spectra were recorded on Bruker (1H, 400 MHz or 600 MHz) spectrometers with...
tetramethylsilane as the internal standard. Chemical shifts (δ) are reported in parts per million (ppm) relative to the reference solvents used. The following abbreviations were used to report the multiplicity: br, broad; m, multiplet; s, singlet; d, doublet; t, triplet; q, quartette; dd, doublet of doublets; dt, doublet of triplets. High-resolution mass spectrometry results were recorded on the Thermo Q-Exactive time-of-flight LC/MS system.

4.1.1. Synthesis of 2-methyl-3-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzonitrile (2)

To a solution of 3-bromo-2-methylbenzonitrile (10.00 g, 51 mmol) in 1,4-dioxane (100 ml), Bis(pinacolato)diboron (15.54 g, 61 mmol), potassium acetate (10.01 g, 102 mmol) and Pd(dppf)Cl2 (1.12 g, 1.53 mmol) were added under nitrogen. The reaction was refluxed for 5 h and was cooled down to room
temperature. The precipitate formed in the mixture was filtered. 50 ml of water was added to the filtrate and then extracted with ethyl acetate (30 ml × 3). The resulting compound was washed with brine and dried over Na2SO4. After filtration and evaporation, the condensation was purified with a silica gel column (petroleum ether/ethyl acetate = 20:1) to yield compound 2 as white solid (11.15 g, 77%). 1H NMR (600 MHz, DMSO-d6) δ (ppm) 7.86 (d, J = 7.2 Hz, 1H), 7.24 (t, J = 6.7 Hz, 1H); 7.38 (q, J = 6.9 Hz, 1H), 2.66 (s, 3H); 13C NMR (151 MHz, DMSO-d6) δ (ppm) 167.16, 165.28, 164.08, 158.60, 155.83, 147.81, 146.45, 145.34, 139.44, 138.47, 136.97, 133.00, 132.66, 129.55, 128.02, 104.47. MS (ESI), m/z: 244.23 [M + H]+.

4.1.2. Synthesis of 3-(2-amino-6-chloropyrimidin-4-yl)-2-methylbenzonitrile (7a)

4,6-Dichloropyrimidin-2-amine (4.55 g, 27.77 mmol), K2CO3 (7.68 g, 55.54 mmol), triethylamine (TEA) (1.24 g, 12.24 mmol) and CuSO4·5H2O (11 mg, 0.045 mmol) and sodium L-ascorbate (13 mg, 0.068 mmol) were added. The reaction was allowed to be stirred for 12 h at 65°C and then concentrated under reduced pressure to remove the solvent. The concentrate was then purified by column chromatography (DCM/MeOH = 50:1 to 25:1) to afford compound 7a. Yield 235.41 mg, 37%. 1H NMR (600 MHz, DMSO-d6) δ (ppm) 7.70 (d, J = 7.2 Hz, 1H), 7.27 (s, 1H), 6.89 (s, 2H), 6.06 (s, 2H), 2.56 (s, 3H); 13C NMR (151 MHz, DMSO-d6) δ (ppm) 166.12, 163.00, 161.69, 157.57, 154.72, 146.37, 145.62, 143.91, 139.44, 138.47, 136.97, 133.00, 132.66, 129.55, 128.02, 104.47. MS (ESI), m/z: 245.15 [M + H]+.

4.1.4. Synthesis of 3-(2-aminopyrimidin-4-yl)-2-methylbenzonitrile (5)

3-(2-Amino-6-ethynylpyrimidin-4-yl)-2-methylbenzonitrile (5) was synthesized using a similar procedure as described above. Yield 419.1722 mg, 78%; m.p. 105°C. 1H NMR (600 MHz, DMSO-d6) δ (ppm) 7.70 (m, 2H), 7.75 (dd, J = 7.7 Hz, 1H), 7.63 (t, J = 7.7 Hz, 1H), 7.52 (t, J = 7.7 Hz, 1H); 7.47 (d, J = 8.1 Hz, 1H), 7.30 (s, 1H), 6.89 (s, 2H), 6.06 (s, 2H), 2.56 (s, 3H); 13C NMR (151 MHz, DMSO-d6) δ (ppm) 166.12, 163.00, 161.69, 157.57, 154.72, 146.37, 145.62, 143.91, 139.44, 138.47, 136.97, 133.00, 132.66, 129.55, 128.02, 127.38, 126.53, 126.36, 126.31, 125.10, 117.40, 112.62, 104.47, 45.52, 17.67. HRMS (ESI) calcd for C23H21N8: [M + H]+ 419.17472, found: 419.17477.

Methyl 6-(4-(2-amino-6-(3-cyano-2-methylphenylimidin-4-yl)-1H-1,2,3-triazol-1-yl)methyl)picolinate (7d), tawny solid. Yield 78%; m.p. 203–204°C; 1H NMR (600 MHz, DMSO-d6) δ (ppm) 8.78 (s, 1H), 8.43 (d, J = 8.5 Hz, 1H), 8.01 (d, J = 8.1 Hz, 1H), 7.97 (d, J = 8.5 Hz, 1H), 7.90 (d, J = 7.7 Hz, 1H), 7.81–7.74 (m, 2H), 7.63 (t, J = 7.5 Hz, 1H), 7.52 (t, J = 7.7 Hz, 1H), 7.47 (d, J = 8.5 Hz, 1H), 7.30 (s, 1H), 6.89 (s, 2H), 6.06 (s, 2H), 2.56 (s, 3H); 13C NMR (151 MHz, DMSO-d6) δ (ppm) 166.12, 163.00, 161.69, 157.57, 154.72, 146.37, 145.62, 143.91, 139.44, 138.47, 136.97, 133.00, 132.66, 129.55, 128.02, 127.38, 126.53, 126.36, 126.31, 125.10, 117.40, 112.62, 104.47, 45.52, 17.67. HRMS (ESI) calcd for C27H23N8a: [M + H]+: 419.1722, found: 419.17472.
Figure 5. Plasma concentration-time profiles of compounds 7f and 7i following oral administration (a) and intravenous administration (b).
4.1.7. Synthesis of 6-((4-(2-amino-6-(3-cyan-2-methylphenyl)pyrimidin-4-yl)-1H,1,2,3-triazol-1-yl)methyl)picolinamidic acid (7h)

Compound 7d (128 mg, 0.30 mmol) were dissolved in a mixed solution of tert butanol (10 ml) and water (5 ml). Then LiOH (18.5 mg, 0.441 mmol) was added and the reaction mixture was continued stirred for 8 h. After reaction, aqueous HCl (1 M) was added to adjust the pH to 5–6. Next, the mixture solution was extracted with ethyl acetate (20 ml × 3). The combined organic layer was washed with brine (30 ml), dried over anhydrous Na2SO4, filtered, and concentrated under reduced pressure. The reaction residue thus obtained was purified by flash column chromatography (DCM/Methanol = 20:1) to afford the target compounds 7i–7l.

4.1.8. General synthetic procedure for compounds 7i–7l

A solution of compound 7d (50 mg, 0.12 mmol) in MeOH (3 ml) and THF (3 ml) was cooled to −10 °C and ammonia or its derivatives (2.4 mmol) was added under nitrogen. The reaction mixture was stirred at 45 °C for 6 h. The reaction progress was monitored by TLC. After completion of the reaction, the reaction mixture was diluted with water (15 ml) and extracted with ethyl acetate (10 ml × 3). The combined organic phase was washed with brine (30 ml), dried over Na2SO4, then concentrated under reduced pressure. The reaction residue thus obtained was purified by flash column chromatography (DCM/Methanol = 50:1 to 20:1) to afford the target compounds 7i–7l.

4.1.9. Synthesis of 2-((6-((2-amino-6-(5-methylfuran-2-yl)pyrimidin-4-yl)-1H)-1,2,3-triazol-1-yl)methyl)pyridin-2-yl)propan-2-ol (17)

Compound 17 was prepared in the same way as 7a from 4-ethyl-6-(5-methylfuran-2-yl)pyrimidin-2-amine (14) with 2-((6-(azidomethyl)pyridin-2-yl)propan-2-ol (16).

4.1.10. Synthesis of 2-((6-((2-amino-6-(3-fluoromethyl)phenyl)pyrimidin-4-yl)-1H,1,2,3-triazol-1-yl)methyl)pyridin-2-yl)propan-2-ol (22)

To a solution of compound 21 (384 mg, 1.00 mmol) and compound 16 (288 mg, 1.50 mmol) in rBuOH (4 ml) and water (4 ml) were added CuSO4·5H2O (25 mg, 0.10 mmol), sodium L-ascorbate (40 mg, 0.20 mmol) and TBAF (1 M in THF) (2.00 ml, 2.00 mmol). The reaction was allowed to be stirred for 12 h at 60 °C and then concentrated under reduced pressure to remove the solvent. The concentrate was then purified by column chromatography (DCM/Methanol = 50:1) to afford compound 22, white solid. Yield 45%; m.p. 188–189 °C; 1H NMR (600 MHz, DMSO-d6) δ (ppm) 8.61 (s, 1H), 7.80 (t, J = 7.8 Hz, 1H), 7.61 (d, J = 7.6 Hz, 1H), 7.46 (s, 1H), 7.14 (d, J = 3.3 Hz, 1H), 7.10 (d, J = 7.5 Hz, 1H), 6.70 (s, 2H), 6.33 (dd, J = 3.3, 1.0 Hz, 1H), 5.80 (s, 2H), 5.21 (s, 1H), 2.40 (s, 3H), 1.38 (s, 6H). 13C NMR (151 MHz, DMSO-d6) δ (ppm) 168.59, 164.25, 158.60, 156.78, 155.20, 153.56, 150.73, 146.48, 138.22, 125.52, 119.87, 118.43, 131.11, 109.37, 72.73, 55.08, 31.03, 14.05. HRMS (ESI) calcd for C20H18N4O2 [M + H]+: 392.18295, found: 392.18250.
4.2. Pharmacology

4.2.1. Cyclic AMP functional assay
CHO-K1/ADORA2A/Gz15 cells expressing human A2A AR and CHO-K1/ADORA2b/Gz15 cells expressing human A2B AR were both constructed by Genscript Biotech Corporation. These two types of cells were cultured in an F12K complete medium (Gibco) at 37 °C under 5% CO2. The 4X test compound (ZM241385, AB928, and target compounds) stock solutions and 4X NECA stock solution were prepared with assay buffer (Hank’s buffered saline solution) for use. The test stock solutions were diluted with different gradients for use. The procedure was exemplified by the cAMP functional assay on the A2A receptor: 10,000 cells/well of CHO-K1/ADORA2A/Gz15 were seeded in a 384-well plate in 20 μl assay buffer. Test compound solutions with different concentrations (2.5 μl) and NECA stock solution (2.5 μl) were added to indicated well of the 384-well plate prepared above and incubated at 37 °C for 30 min. Then, 10 μl of detection reagent (cAMP-d2 and anti-cAMP-Eu3+) was added into each well of the plate and incubated at room temperature for 1 h. The plate was transferred into the PHERA Star for HTRF detection and the data (ratio 665/620) was collected at the wavelength of 665 nm and 620 nm. The inhibition was calculated according to the formula: inhibition (%)=((R0−R)/R0)×100%, where R0 is the ratio 665/620 value in the presence of target compounds, R is the ratio 665/620 value during the blank assay and R0 is the ratio 665/620 value in the presence of ZM241385 at a concentration of 1 μM.

4.2.2. A2a AR binding assay
The target compounds were tested to evaluate their affinity for the A2A AR on HEK-293 cell membranes expressing human A2A AR. [3H]-ZM241385 was used as a radioligand. The assay buffer (50 mM Tris-HCl, pH 7.4; 10 mM MgCl2; 1 mM EDTA; 1 g/ml adenosine deaminase), wash buffer (50 mM Tris-HCl, pH 7.4; 1 μg/ml bacitracin), and 0.5% PEI solution were prepared and stored at −20°C. The absolute bioavailabilities of synthetic compounds (7f, 7i) were studied with pooled human (male) liver microsomes solution (20 mg/ml) and male Sprague–Dawley rats pooled liver microsomes solution (20 mg/ml) purchased from Research Institute for Liver Diseases (Shanghai) Co. Ltd. Both liver microsomes solutions (140 μl) were diluted by 3948 μl of water and 280 μl of phosphate buffered saline. The assay procedure: 640 μl of diluted liver microsomes solution was added to a solution of compound working solution (16 μl, 25 μM in DMSO) and the mixed solution was incubated at 37 °C for 5 min. Then 160 μl of NADPH generating system was added and take out 100 μl from the mixed solution was added 200 μl of acetonitrile solution to stop the reaction at the set incubation time (0 min, 10 min, 20 min, 30 min, 40 min, 50 min, and 60 min). All stopped reaction solutions were vortex mixed (3 min) and centrifuged (10,000 rpm, 4 °C for 10 min). Then 50 μl of the supernatant was analysed by UPLC-MS/MS system (details of the analytical method see Supporting information).

4.3. Molecular docking
The crystal structure of hA2A AR in complex with antagonist ZM241385 (PDB ID: 5IU4) was retrieved from the Protein Data Bank. The primary sequence of hA2A AR was obtained from the NCBI/UNIPROT online database (www.ncbi.nlm.nih.gov/protein/P29275.1). Based on the primary sequence, the homology model of hA2B AR was built using the Swiss Model program from a hA2A AR crystal structure as the template (PDB ID: 6PS7) and has been checked using the Ramachandran plot application within Discovery Studio 2017 R2. The molecular docking was performed under the CDOCKER protocol of Discovery Studio. First, the 3D structure of 7i was generated and the hydrogenation, dehydration, and CHARMM force field of the protein were executed. Then, the protein was remodelled by removing the antagonist and the 3D molecule of 7i was placed at the active site for molecular docking. Next, the 2D and 3D predicted binding models of compound 7i in hA2A AR and hA2B AR were generated.

4.4. Metabolic stability in human and rat liver microsomes
Metabolic stabilities of compounds 7f, 7i, and AB928 in liver microsomes were studied with pooled human (male) liver microsomes solution (20 mg/ml) and male Sprague–Dawley rats pooled liver microsomes solution (20 mg/ml) purchased from Research Institute for Liver Diseases (Shanghai) Co. Ltd. Both liver microsomes solutions (140 μl) were diluted by 3948 μl of water and 280 μl of phosphate buffered saline. The assay procedure: 640 μl of diluted liver microsomes solution was added to a solution of compound working solution (16 μl, 25 μM in DMSO) and the mixed solution was incubated at 37 °C for 5 min. Then 160 μl of NADPH generating system was added and take out 100 μl from the mixed solution was added 200 μl of acetonitrile solution to stop the reaction at the set incubation time (0 min, 10 min, 20 min, 30 min, 40 min, 50 min, and 60 min). All stopped reaction solutions were vortex mixed (3 min) and centrifuged (10,000 rpm, 4 °C for 10 min). Then 50 μl of the supernatant was analysed by UPLC-MS/MS system (details of the analytical method see Supporting information).

4.5. Pharmacokinetics assay
Animal experiments were performed according to the institutional ethical guidelines on animal care and approved by the Institute Animal Care and Use Committee at Binzhou Medical University.

The absolute bioavailabilities of synthetic compounds (7f, 7i) were studied in female Bal b/c mice (Jinan Pengyue Experimental Animal Breeding Co., Ltd.). The mice were divided into four groups each having six mice and were collected at crossover time points, with three mice at each point. Pharmacokinetics (PK) was evaluated after a single dose of 30 mg/kg oral gavage (Oral) or 10 mg/kg intravenous (i.v.) administration. After oral administration and i.v. injection, blood samples were obtained from the suborbital veniplex at pre, 15 min, 30 min, 1 h, 2 h, 4 h, 6 h, 8 h, 12 h, 24 h (Oral) and pre, 5 min, 15 min, 30 min, 1 h, 2 h, 4 h, 6 h, 8 h, 12 h, 24 h (i.v.). Approximately 200 μl of blood was collected at each time point. All blood samples were put into plastic microcentrifuge tubes containing Heparin-Na as an anticoagulant. Microcentrifuge tubes with blood samples and anticoagulant were inverted several times for proper mixing of the tube contents to centrifugation for plasma. Plasma samples will be centrifuged at 12,000 rpm for 8 min at 4 °C to obtain the supernatant. The serum sample (25 μl) was treated with acetonitrile (100 μl), after which the mixture was vortex-mixed for 8 min and centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatant layer was collected and then 50 μl of supernatant was injected for the UPLC-MS/MS analysis (details of the analytical method see Supporting information).

Disclosure statement
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