ABSTRACT: Ischemic stroke is a complex systemic disease characterized by high morbidity, disability, and mortality. The activation of the presynaptic adenosine $A_2A$ and $A_1$ receptors modifies a variety of brain insults from excitotoxicity to stroke. Therefore, the discovery of dual $A_2A/A_1$ adenosine receptor (AR)-targeting therapeutic compounds could be a strategy for the treatment of ischemic stroke. Inspired by two clinical phase III drugs, ASP-5854 (dual $A_2A/A_1$ AR antagonist) and preladenant (selective $A_2A$ AR antagonist), and using the hybrid medicinal strategy, we characterized novel pyridone-substituted triazolopyrimidine scaffolds as dual $A_2A/A_1$ AR antagonists. Among them, compound 1a exerted excellent $A_2A/A_1$ AR binding affinity ($K_i = 5.58/24.2 \text{ nM}$), an antagonistic effect ($IC_{50} = 5.72/25.9 \text{ nM}$), and good metabolic stability in human liver microsomes, rat liver microsomes, and dog liver microsomes. Importantly, compound 1a demonstrated a dose–effect relationship in the oxygen-glucose deprivation/reperfusion (OGD/R)-treated HT22 cell model. These findings support the development of dual $A_2A/A_1$ AR antagonists as a potential treatment for ischemic stroke.

KEYWORDS: Ischemic stroke, dual $A_2A/A_1$ AR antagonist, pyridone-substituted triazolopyrimidine
anti-CTLA-4 mAb. Moreover, the discovery and development of dual A2A/AR therapeutic compounds is an attractive and alternative therapeutic strategy for improving the in vivo efficacy of a single target. For example, AB928, a potent and selective dual A2A/A2B AR antagonist discovered by Arcus Biosciences, is currently undergoing clinical trials in multiple cancer settings.

Ischemic stroke is a complex systemic disease characterized by high morbidity, disability, and mortality. Increasing substantial evidence has shown a protective role for A2A AR antagonists in striatal and nigral neurons through the prevention of glutamate-dependent neuronal death, thereby reducing cortical damage in a variety of ischemic stroke models. In A2A AR knockout (KO) mice, transient focal ischemia causes less neuronal damage compared with that in wild-type (WT) mice. The selective A2A AR antagonist SCH58261 reduced ischemic brain damage in an adult rat model of focal cerebral ischemia. Meanwhile, the activation of the A1 AR was able to induce ischemic damage protection and the reduction of both reactive and proliferative microglia/macrophages after experimental stroke in rats. These results demonstrate that the activation of the presynaptic A2A and A1 ARs modifies a variety of brain insults from excitotoxicity to stroke.

Owing to our interest in ARs and the field of ischemic stroke, we set out to design and synthesize novel dual A2A/A1 AR antagonists based on the crystal structures of A2A AR (PDB: 3EML) and A1 AR (PDB: SEUN) complexes. We herein report the discovery and characterization of a new chemotype of dual A2A/A1 AR antagonists with a pyridone-substituted triazolopyrimidine scaffold, in which compound 1a demonstrated a remarkable dose–effect relationship in the oxygen-glucose deprivation/reperfusion (OGD/R)-treated HT22 cell model.

Our initial design was inspired by two known clinical phase III drug candidates, preladenant (selective A2A AR antagonist), with a triazolopyrimidine scaffold, and ASP-5854 (dual A2A/A1 AR antagonist), with a pyrazine scaffold. Using insight from preladenant crystal structures with an A2A AR and an A1 AR, we noticed that the primary amide (ring A) and the triazole (ring B) with furan rings in preladenant established two bidentate hydrogen-bonding interactions with Glu169 and Asn253 (Figure 2A,B) with similar binding modes. However, preladenant is just a selective A2A AR antagonist, suggesting that the triazolopyrimidine scaffold was a key pharmacophore as the selective A2A AR antagonist. The primary amide in ASP-5854 also formed a bidentate hydrogen-bonding interaction with Asn253, in which compound 1a was a key pharmacophore. Moreover, the pyridone in ASP-5854 formed an additional hydrogen bond with Thr90 in the A1 AR, which may be a key pharmacophore as the A1 AR antagonist. On the basis of these analyses, we intended to exploit the hybrid drug design approach to access all five aforementioned interactions with the goal of identifying a novel chemical scaffold dual A2A/A1 AR antagonist with better drug-like properties. Therefore, we designed and synthesized a series of compounds with the novel pyridone-substituted triazolopyrimidine chemotype and carried out a systematic study of structure–activity relationships (SARs).

As shown in Scheme 1, the synthetic strategy of 1a–1i involved a three-step sequence, including a nucleophilic substitution reaction, Dimroth rearrangement, and a Suzuki coupling process, starting from commercially available aryl formamide derivatives. Initially, the nucleophilic substitution of 5-bromo-4-chloropyrimidin-2-amine (3) by aryl formamide 2a–2i at 120 °C in n-butanol proceeded smoothly to deliver compounds 4a–4i. The subsequent Dimroth rearrangement of compounds 4a–4i was conducted in the presence of N,O-bis(trimethylsilyl) acetamide (BSA) and hexamethyldisilazane (HMDS) at 120 °C to give the desired cyclization triazolopyrimidine compounds 5a–5i. Finally, compounds 5a–5i were coupled to pyridone boronic esters (6) to afford...
the final products 1a−1i in an acceptable yield (4.1−6.5%) over three steps.

The binding affinity of the synthesized pyridone-substituted triazolopyrimidine derivatives (1a−1i) toward the A2A and A1 ARs, along with A2B and A3 ARs, was evaluated in competitive binding experiments using membrane preparation of the human recombinants A1, A2A, and A3, and the A2B AR overexpressed from CHO, HeLa, and HEK-293 cells, respectively. [3H]DPCPX (A1), [3H]ZM241385 (A2A), [3H]DPCPX (A2B), and [3H]NECA (A3) were used as radioligands. The binding affinity data of synthesized compounds are listed in Table 1, with pyrazine antagonist 1a with a furan ring as the Ar group exhibited the most excellent binding affinity against the A2A AR (Ki = 5.58 nM against the A2A AR and 24.2 nM against the A1 AR) and a high degree of selectivity for the A2B AR (A2B/A2A 88-fold) and the A3 AR (A2B/A3 78.0 nM against the A2B AR and 24.2 nM against the A1 AR and a high degree of selectivity over the A2A AR). The binding affinity data of synthesized compounds are in Table 1, with pyrazine antagonist 1a with a furan ring as the Ar group exhibited the most excellent binding affinity (Ki = 5.58 nM against the A2A AR, 24.2 nM against the A1 AR, and a high degree of selectivity for the A2B AR (A2B/A2A 88-fold) and the A3 AR (A3/A2A 1575-fold), respectively. Compounds 1b (pyridine as the Ar group) and 1c (thiazole as the Ar group) showed moderate binding affinity against the A2A AR (Ki = 62.4 nM) and comparable binding activity against the A1 AR (Ki = 84.6 nM), along with good selectivity over the A2B and A3 ARs. Interestingly, compound 1d (5-methylthiazole as the Ar group) displayed the most potent binding affinity data, with a Ki value of 21.9 nM against the A1 AR and two-fold selectivity over the A2A AR, which can be used as a lead for the further optimization of selective A1 AR antagonists. However, compounds 1e−1i showed less binding affinity against A1 to A3 ARs compared with compounds 1a−1d because of the introduction of a methyl group beside the heteroatom, which may increase the steric hindrance and affect the binding to the target cavity of A1 to A3 ARs. These results suggested that the introduction of heteroatoms in the Ar group was crucial for binding to the A2A AR and increased its affinity, and furan as the Ar group was the most potent. Conversely, the introduction of a methyl group beside the heteroatom on the Ar group was fatal for binding to the target due to the steric hindrance.

Furthermore, the calcium flux functional experiments were carried out to assess the antagonistic/agonistic activity of the most potent compound 1a at the A2A and A1 ARs, along with A2B and A3 ARs. The functional assay data IC_{50} for 1a shown in Figure 3 indicated that the excellent antagonist activity of 1a was consistent with its binding affinity, whereas the agonist activity of 1a was negligible. Many studies have proven that neuron apoptosis is involved in the pathological process of ischemia injury. Thus the OGD/R model (in vitro ischemic model) was used to damage HT22 cells to simulate ischemic injury to investigate the effect of A2A/A1 AR antagonist compound 1a on HT22 cell damage. First, we investigated the effect of compound 1a on cell apoptosis induced by OGD/R. The results demonstrated that OGD/R significantly induced apoptosis of HT22 cells, and compound 1a reversed, in a concentration-dependent manner, the up-regulation of pro-apoptotic genes such as cleaved caspase-3, cleaved caspase-9, cleaved PARP1, p53, and Bax in OGD/R-treated HT22 cells (Figure 4A). Notably, immunofluorescence analyses revealed that the antiapoptotic gene Bcl-2 staining was enhanced by compound 1a (Figure 4B). Likewise, the mRNA expression in the apoptosis markers (p53, Bax, Bcl-2) was consistent with the protein expression (Figure 4C). These results provided support that compound 1a protected against cell apoptosis.

Currently, there is strong evidence that inflammatory processes may contribute to secondary brain damage after ischemic stroke. Indeed, inflammation modulators including iNOS, COX-2, and VCAM-1 were induced by OGD/R; on the contrary, increased inflammation mediators were significantly inhibited by compound 1a treatment (Figure 5A). Consistent with these findings, immunofluorescence staining revealed a lack of COX-2 in compound-1a-treated HT22 cells as compared with that in OGD/R-treated HT22 cells (Figure 5B). Recent findings identified that NLPR3 inflammasomes play a major role in neuronal cell death in stroke and further suggested that targeted inflammasome assembly and activity may ameliorate ischemic injury. We found that OGD/R robustly induced the expression of inflammasome protein caspase-1 (p20) and mature pro-inflammatory cytokines IL-18
and IL-1β in HT22 cells; in turn, compound 1a reduced caspase-1 (p20), IL-18, and IL-1β expression (Figure 5C). Likewise, the mRNA expression in inflammation markers Nos2, Vcam-1, and II1b was consistent with the protein expression (Figure 5D). These results provide further support that compound 1a protected against neuron OGD/R injury.

The metabolic stability is a prime consideration when developing a candidate (Table 2). The in vitro metabolic stability of compound 1a was measured using human liver microsomes (HLMs), rat liver microsomes (RLMs), mouse liver microsomes (MsLMs), dog liver microsomes (DLMs), and monkey liver microsomes (MkLMs). Compound 1a displayed good metabolic stability with a half-life of 77.4, 56.0, and 83.9 min along with an intrinsic clearance (CL) of 1601.7 and 128 mL/min/kg in the MsLMs and HLMs, respectively. After 60 min, 58.3, 48.6, and 60.6% of compound 1a remained in the HLMs, RLMs, and DLMs, respectively. However, compound 1a displayed less metabolic stability with a half-life of 3.4 and 14.6 min along with an intrinsic clearance (CL) of 1601.7 and 128 mL/min/kg in the MsLMs and MkLMs, respectively. In addition, compound 1a showed moderate brain penetration (B/P ratio = 0.22) (Table S3), which is suitable for the lead compound of ischemic stroke.

Molecular docking modeling was performed to interpret the dual A2A/A1 AR binding affinity of compound 1a at the molecular level. The binding modes of compound 1a at the A2A and A1 AR cavities were analyzed by docking simulations using the Autodock software package, with the crystal structures of the A2A and A1 AR complexes as templates, respectively. The docking results (Figure 6) revealed that compound 1a adopted the general binding mode at both the A2A and A1 AR binding sites. In this binding mode, the pyridone-substituted triazolopyrimidine scaffold was positioned in the depth of the binding pocket and underwent a π–π interaction with the Phe residue (Phe168 in the A2A AR).
Phe171 in the A₁ AR). In addition, compound 1a formed five hydrogen bonds with the A₂A AR (Glu169, Asn253, and His278), whereas it formed only two hydrogen bonds with the A₁ AR (Asn253). Therefore, compound 1a adopted a much more favorable binding pose at the A₂A AR cavity than at the A₁ AR cavity. Moreover, the Autodock docking results also indicated that the binding pose was associated with a better docking score at the A₂A AR (−9.02 kcal/mol, Kᵢ = 0.24 μM) than at the A₁ AR (−7.79 kcal/mol, Kᵢ = 1.94 μM). Hence, the molecular docking results explained the A₂A AR affinity and the slight selectivity over the A₁ AR (4.4-fold) of compound 1a.

In the present study, we designed and synthesized a novel pyridone-substituted triazolopyrimidine scaffold dual A₂A/A₁ AR antagonist using a computer-aided rational drug design approach along with a hybrid medicinal strategy, inspired by two phase III drugs, ASP-5854 (dual A₂A/A₁ AR antagonist) and preladenant (selective A₂A AR antagonist) and preladenant (selective A₂A AR antagonist). In vitro evaluations of the A₁, A₂A, A₁β, and A₂A AR binding assays for the synthesized compounds showed promising results. Among them, compared with ASP-5854, the most potent compound 1a showed a better clog P and comparable A₂A/A₁ AR binding affinity (Kᵢ = 5.58/24.15 nM). In addition, compound 1a showed excellent solubility at pH 1.2 (1.99 mg/mL), which is suitable for oral administration after some rational modification. Moreover, compound 1a showed an excellent antago-nistic effect (IC₅₀ = 5.72/25.93 nM), moderate brain penetration (B/P ratio = 0.22), and good metabolic stability with T₁/₂HLM = 77.4 min, T₁/₂RLM = 56 min and T₁/₂DLM = 83.9 min. Importantly, compound 1a demonstrated a remarkable dose–effect relationship in the OGD/R-treated HT22 cell model, including the reduction of HT22 cells apoptosis and an alleviation of inflammatory modulator (iNOS, COX-2, and VCAM-1) and inflammatory cytokine (p20, IL-1β, Nos2, Vcam-1, and II1b) release. With these encouraging results, we anticipate that this novel pyridone-substituted triazolopyrimidine scaffold could be an excellent starting point for the further development of dual A₂A/A₁ AR antagonists to benefit the field of ischemic stroke. The current effort is focused on further improving the potency along with good pharmacokinetics and pharmacodynamics both in vivo and in vitro, and these findings will be reported in due course.

**ASSOCIATED CONTENT**

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsmedchemlett.1c00599.

Experimental procedures and characterization for all final compounds and descriptions of in vitro studies (PDF)

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**Notes**

The authors declare no competing financial interest.

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**ABBREVIATIONS**

AR, adenosine receptor; OGD/R, oxygen-glucose deprivation/reperfusion; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase 2; VCAM-1, vascular cell adhesion molecule 1; p20, protein 20; IL-18, interleukin-18; IL-1β, interleukin-1β; Nos2, nitric oxide synthase 2; GPCR, G-protein-coupled receptor; cAMP, cyclic adenosine monophosphate; PD, Parkinson’s disease; HD, Huntington’s disease; AD, Alzheimer’s disease; KO, knockout; WT, wild-type; SAR, structure–activity relationship; BSA, N,O-bis(trimethylsilyl) acetamide; HMDS, hexamethyldisilazane; NLRP3, NLR family pyrin domain containing 3; HLM, human liver microsome; RLM, rat liver microsome; MsLM, mouse liver microsome; DLM, dog liver microsome; CL, clearance

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