Bioorganic & Medicinal Chemistry Letters 26 (2016) 2719–2723

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

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

Discovery of indirubin derivatives as new class of DRAK2 inhibitors from high throughput screening

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ARTICLE INFO

Article history:
Received 23 December 2015
Revised 27 March 2016
Accepted 31 March 2016
Available online 1 April 2016

Keywords:
Indirubin
DRAK1
DRAK2
Kinase
Inhibitor

ABSTRACT

DRAK2 is a serine/threonine kinase belonging to the death-associated protein kinase (DAPK) family and has emerged as a promising drug target for the treatment of autoimmune diseases and cancers. To identify small molecule inhibitors for DRAK2, we performed a high throughput screening campaign using in-house chemical library and identified indirubin-3′-monoximes as novel class of DRAK2 inhibitors. Among the compounds tested, compound **16** exhibited the most potent inhibitory activity against DRAK2 (IC_{50} = 0.003 μM). We also propose that compound **16** may bind to the ATP-binding site of the enzyme based on enzyme kinetics and molecular docking studies.

DRAK2 (DAP kinase-related apoptosis-inducing protein kinase 2) is a serine/threonine kinase belonging to the death-associated protein kinase (DAPK) family.[1] All members of the DAPK family induce apoptosis when expressed ectopically in various cell types.[2] However, the role of DRAK2 in the regulation of apoptosis is still controversial and its ability to induce apoptosis seems to depend on the cellular context.[3,4] DRAK2 expression is highly enriched in B and T cells suggesting the possible involvement of the enzyme in immunological processes.[5] Consistent with this idea, DRAK2-deficient knock-out mice develop resistance to autoimmune diseases including EAE (experimental autoimmune encephalomyelitis) and type I diabetes.[6,7] In addition, it has been reported that DRAK2 mediated signaling plays an important role in graft rejection after organ transplantation.[8] Thus, pharmacological inhibition of DRAK2 could be a promising strategy to achieve transplant maintenance without causing the unwanted immunosuppression. DRAK2 has also been suggested to play an important role in tumorigenesis; DRAK2 expression is highly elevated in basal-like and HER2-enriched human breast tumors and loss of DRAK2 expression resulted in the inhibition of tumor growth in a xenograft model.[9]

Despite the increasing evidence for DRAK2 as a promising drug target, only a few example of small molecule inhibitor development has been reported in the literature. Marvaldi et al. reported the effects of a DRAK2 inhibitor, SC82510, on axon outgrowth.[10] SC82510 was derived from the previously reported casein kinase inhibitors and reported to have the ability to promote axon branching when treated to neuronal cells in vitro. However, the exact chemical structure of the compound has not been disclosed in the paper. A series of 5-arylthieno[2,3-*b*]pyridines reported by Leonczak et al. has been found to exhibit promising inhibitory activity for DRAK2 with the K_d value of 8 nM for the most potent one.[11] Recently, Gao et al. also reported the discovery of a thieno[2,3-*b*]pyridine derivative as a potent DRAK2 inhibitor displaying a K_d value of 9 nM.[12] The identification of DRAK2 inhibitors with novel scaffolds can be used as a starting point for further optimization in drug discovery programs and also very useful for understanding the role of the enzyme in cellular signal transduction.

Indirubin is the active ingredient of Danggui Longhui Wan, a mixture of plants that is used in traditional Chinese medicine to

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http://dx.doi.org/10.1016/j.bmcl.2016.03.111
0960-894X/

treat chronic diseases. It has been shown to possess a variety of biological activities including antimitotic and antitumor properties and reported to have inhibitory activity for protein kinases such as CDK2,[13–17] GSK-3β[18,19] and FLT3.[20] The rather loose selectivity of indirubin allows us to utilize it as a useful starting point to develop the inhibitors for various kinases. Thus, it is not surprising that some indirubin analogues have been identified to be DRAK2 inhibitors in this study. Here, we investigated the effects of indirubin analogues on the activity of this novel kinase DRAK2.

In an effort to discover small molecules to inhibit DRAK2, an inhibitor screen was performed using more than 11,000 compounds from in-house library by in vitro kinase assay using the purified recombinant DRAK2 protein as an enzyme source.[21] The average Z' value over the entire primary screen was found to be 0.60 ± 0.07, indicating the assay is robust and reproducible. A total of 56 compounds showing more than 70% inhibition were selected as primary actives for further confirmation. The activity of 56 compounds was re-confirmed in triplicate and finally 41 compounds were nominated as hits. We determined the dose–response curves of these 41 compounds and analyzed their structures to classify them depending on the scaffolds. Many of them were classified into several clusters with unique scaffolds while others are present as singlets or doublets. One of the actives from this high throughput screening campaign is a series of indirubin (**1**) or indirubin-3′-monoxime (**2**) derivatives (Fig. 1).

To compare the inhibitory potency of the indirubin analogues with the previously reported DRAK2 inhibitors, we have chosen the compound having a thieno[2,3-*b*]pyridine moiety reported by Gao et al.[12] as a reference for DRAK2 inhibition. The reference compound, *N*-(5-(3,4 dimethoxyphenyl)thieno[2,3-*b*]pyridin-3-yl)-cyclohexanecarboxamide, was synthesized according to the previously reported procedures.[12] The IC_{50} values of the compound from in vitro kinase assay were found to be 0.024 and 0.044 μM, respectively, for DRAK1 and DRAK2, which are comparable to previously reported K_d values for the compound.

A synthetic chemistry into indirubins is outlined in Scheme 1. 5-Nitroindoline-2,3-dione and 1*H*-indol-3-yl acetate were condensed in the presence of sodium carbonate in methanol. The nitro adduct was reduced into the aniline homolog using iron powder. The aniline was coupled with various acid chlorides and then treated with hydroxylamine to provide indirubin DRAK2 inhibitors.[22]

Initial efforts were made to explore the structure–activity relationship (SAR) for a representative selection of indirubin analogues and the results are summarized in Table 1. The compounds having the substitutions at R^1 and/or R^3 positions were generally inactive in inhibiting DRAK2 activity (**8**–**12**). Instead, the introduction of an oxime group at R^4 position increased the inhibitory potency of the compounds significantly (compare **1** with **2**). Also, presence of a nitrogen atom at R^2 position seems to be important for the interaction of the compound with the active site of DRAK2 (compare **1** with **7** and **2** with **14**). This preliminary SAR led us to investigate the effects of the substituents at R^2 position of indirubin scaffold on DRAK2 activity in the presence of oxime group at R^4 position.

A series of 5-*N*-acyl indirubin-3′-monoxime analogues were evaluated for DRAK2 kinase inhibitory activity (Table 2). In general, substitution of the aliphatic groups at R^5 position (**15**–**19**) markedly increased the inhibitory activity of the compounds for DRAK2. The most potent one (**16**) was found to have an IC_{50} of 0.003 μM. In contrast, the compounds having a bulky benzoyl group at the position (**22**–**33**) exhibited only moderate inhibitory activity, strongly indicating that the active site cavity of DRAK2 near the R^5 position is relatively small and not enough to accommodate bulky side chains of the inhibitor.

From the SAR studies in Tables 1 and 2, we found that the oxime group at R^4 position and small aliphatic groups at R^5 position are essential for increasing the inhibitory activity of indirubins against

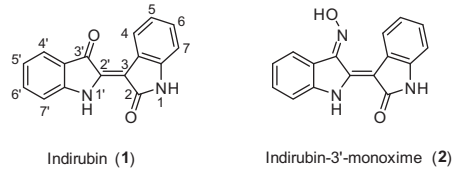
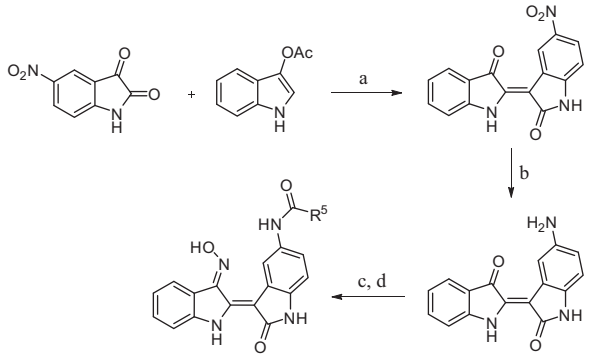
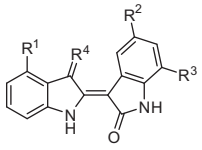


Figure 1. Structures of indirubin and indirubin-3′monoxime.



Scheme 1. Reagents and conditions: (a) Na_2CO_3, MeOH, rt; (b) Fe, NH_4Cl, EtOH/H_2O, 60 °C (2 steps 11%); (c) R^5COCl, pyridine, 0 °C; (d) $H_2NOH{\cdot}HCL$, pyridine, reflux (2 steps 40%).

Table 1
Inhibition of DRAK2 activity by indirubin analogues



Compound	R^1	R^2	R^3	R^4	IC_{50} (μM)[a],[23] DRAK2
1	H	H	H	O	>10
2	H	H	H	NOH	0.71
3	H	Cl	H	O	>10
4	H	F	H	O	>10
5	H	I	H	O	6.2
6	H	CH_3	H	O	>10
7	H	NH_2	H	O	1.4
8	Br	$-NO_2$	H	O	>10
9	Br	H	H	O	>10
10	H	Cl	$-CH_3$	O	>10
11	H	$-CH_3$	$-CH_3$	O	>10
12	H	Cl	$-CH_3$	NOH	>10
13	H	F	H	NOH	0.62
14	H	NH_2	H	NOH	0.25

[a] Values are the mean of two independent experiments.

DRAK2. However, structural diversity of the compounds in Tables 1 and 2 does not seem to be enough to deduce a full scope of SAR for the inhibition. Further studies should be done by preparing and evaluating 5-*N*-acyl indirubin-3′-monoxime derivatives having a variety of substituent groups at the indirubin scaffold.

Selectivity of the potent DRAK2 inhibitors (**15**–**20**) was profiled against phylogenetically related other kinases of DAPK family (Table 3). All the tested compounds seem to exhibit only moderate inhibitory activity for DAPK1, DAPK2 and DAPK3, whereas DRAK1 is relatively strongly inhibited by the compounds. Although compound **16** seems to be specific for DRAK2, most of the indirubin-oxime analogues tested in this study is considered to be dual inhibitors for DRAK1 and 2.