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Discovery of 5-(*N*-hydroxycarbamimidoyl) benzofuran derivatives as novel indoleamine 2,3-dioxygenase 1 (IDO1) inhibitors

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ABSTRACT

Human indoleamine 2,3-dioxygenase 1 (*h*IDO1) and tryptophan dioxygenase (*h*TDO) are rate-limiting enzymes in the kynurenine pathway (KP) of L-tryptophan (L-Trp) metabolism and are becoming key drug targets in the combination therapy of checkpoint inhibitors in immunoncology. To discover a selective and potent IDO1 inhibitor, a structure–activity relationship (SAR) study of *N*-hydroxybenzofuran-5-carboximidamide as a novel scaffold was investigated in a systematic manner. Among the synthesized compounds, the *N*-3-bromophenyl derivative **19** showed the most potent inhibition, with an IC_{50} value of 0.44 μM for the enzyme and 1.1 μM in HeLa cells. The molecular modeling of **19** with the X-ray crystal structure of IDO1 indicated that dipole-ionic interactions with heme iron, halogen bonding with Cys129 and the two hydrophobic interactions were important for the high potency of **19**.

L-Tryptophan (L-Trp) metabolism follows three significant pathways: the kynurenine pathway (KP), the serotonin production pathway, and the direct transformation to indole derivatives by intestinal microorganisms.[1] In mammalian cells, most L-Trp molecules are metabolized mainly via the KP, which is known to control immunoregulatory functions, cell proliferation suppression, reproduction and the central nervous system. In the KP, L-Trp is converted first into *N*-formyl-L-kynurenine by the rate-limiting enzymes, indoleamine 2,3-dioxygenase 1 and 2 (IDO1 and IDO2) or tryptophan 2,3-dioxygenase (TDO), which are attractive targets in immunotherapeutics. Indeed, the overexpression of these enzymes is commonly found in various types of cancers, such as glioblastoma, melanoma, lung cancer, ovarian cancer and colon cancer, in which the prognosis and survival rates are poor.[2]

The metabolic depletion of L-Trp and the production of kynurenine metabolites by IDO1/IDO2 and TDO can cause immunosuppression by suppressing effector T cells and NK cells, activating circulating T regulatory cells (Tregs), promoting the differentiation of CD4+ T cells to Tregs and stimulating the immunosuppressive microenvironment through the secretion of soluble mediators. Therefore, IDO1/IDO2 and TDO inhibition is a promising new cancer immunotherapeutic strategy that effectively enhances antitumor immunity.[3] Clinical trials on the immunotherapy of IDO inhibition have been conducted as either a single agent or combinatorial regimen with monoclonal antibodies (mAbs) (anti-cytotoxic *T*-lymphocyte-associated protein 4 (CTLA-4) mAb and anti-programmed cell death protein 1(PD-1)/programmed death-ligand 1 (PD-L1) mAb) or radiation, but there has been no remarkable success to date.

Among the IDO1-selective inhibitors, epacadostat (INCB-024360) (**1**), navoximod (GDC-0919) (**2**), and linrodostat (BMS-986205) (**3**) showed high affinity for the IDO1 enzyme or its apo-form (Fig. 1).[4–8] Epacadostat has been the leading molecule in the clinical development of IDO1 inhibitors but failed to demonstrate its efficacy in a phase 3 clinical trial in patients with unresectable or metastatic melanoma. Epacadostat in combination with pembrolizumab and the anti-PD-1 mAb also did not improve progression-free survival or overall survival compared with placebo plus pembrolizumab, leading to the early termination of the ECHO-301/Keynote-252 trial.[9]

Although the development of IDO1 inhibitors has lost momentum due to these failures, translational and clinical trials to confirm new possibilities are still actively underway. Moreover, it was observed increased expression of IDO and PD-L1 along with penetration of Treg in tumor microenvironment,[10] and it is known that PD-1 or CTLA4

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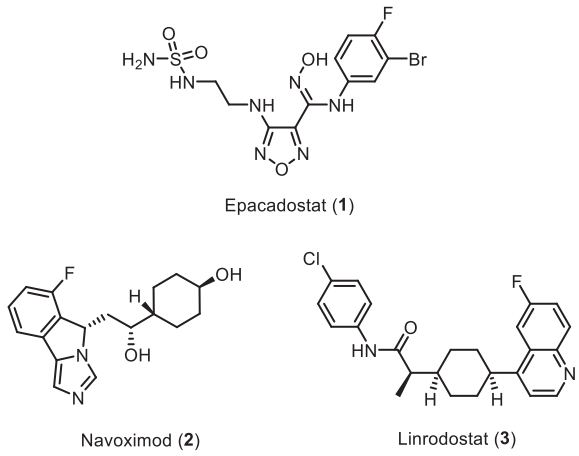


Fig. 1. IDO-1-selective inhibitors.

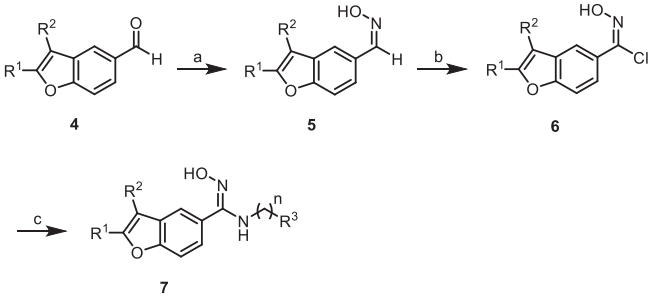
blockades can induce upregulation of IDO1, which can cause an immunosuppressive environment around tumor.[2,10] Therefore, the development of IDO inhibitors still has significant value in that immunotherapy combined with these inhibitors may produce synergistic effects with excellent anticancer activity and highly favorable safety profiles.

In this study, *N*-hydroxybenzofuran-5-carboximidamide as a novel scaffold designed from epacadostat was investigated as a novel IDO1 selective inhibitor. The structure–activity relationship (SAR) was studied by varying the *N*-substituent in *N*′-hydroxyamidine and the 2-substituent in benzofuran to evaluate its inhibition in enzyme and cell-based assays. Molecular modeling of selective inhibitors was performed to identify the binding mode in the active site.

The final 5-(*N*-substituted-*N*′-hydroxycarbamimidoyl) benzofuran derivatives were generally synthesized by a three-step synthesis process, which included aldoxime formation from the corresponding benzofuran aldehyde, α-chlorination of aldoxime and nucleophilic substitution of amine to form *N*-hydroxyamidine (Scheme 1).

Commercially available benzofuran-5-carboxaldehyde **4** was converted to the corresponding aldoxime **5** by treating hydroxylamine and resulted in a very high yield. α-Chlorination on aldoxime **5** was performed by electrophilic addition of *N*-chlorosuccinimide (NCS) to provide oxyimidic chloride **6**. It should be noted that chlorination was conducted under temperature control by maintaining 50 °C, otherwise this reaction resulted in a low yield. Initially, oxyimidic chloride was used directly for the next step without further purification but provided intractable side products. Therefore, to obtain the final compound with high purity, oxyimidic chloride **6** was purified and then reacted with the corresponding primary amines to afford the final *N*-hydroxyamidine **7**.

For the syntheses of 2-alkoxymethyl benzofuran derivatives, 4-cyanophenol **8** was iodinated by treating with iodine and ammonia in MeOH to produce 4-hydroxy-3-iodobenzonitrile **9**. Benzonitrile **9** was reacted with propargyl alcohol in the presence of 10 mol % Pd(II) catalyst and diisopropyl amine to undergo the sequential two-step reaction in which the Sonogashira coupling of **9** with propargyl alcohol formed an acetylene intermediate and then cyclized into 2-(hydroxymethyl)benzofuran **10**.[11] Thereafter, the hydroxymethyl group of **10** was either protected by the *tert*-butyldimethylsilyl (TBS) group or alkylated with a methyl or cyclopropylmethyl group to produce **11**. The nitrile group of **11** was reduced to the corresponding aldehyde **12**, which was converted to the final *N*-hydroxyamidine **13** in three steps by following the same route described in Scheme 1. For the 2-(hydroxymethyl)benzofuran derivatives, the TBS group of **13** was deprotected using tetrabutylammonium fluoride to produce the final **14** (Scheme 2).

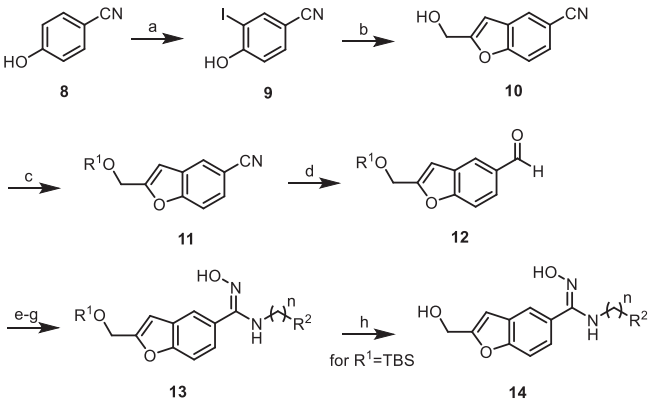


Scheme 1. Synthesis of the 5-(*N*-hydroxycarbamimidoyl)benzofuran derivatives *Reagents and conditions*: (a) NEt_3, $NH_2OH \cdot HCl$, CH_2Cl_2, r.t., 16 h; (b) NCS, DMF, 50 °C, 3 h; (c) $R^3(CH_2)_nNH_2$, THF, r.t., 16 h.

The inhibition of synthesized compounds for IDO1 was evaluated by measuring the kynurenine concentration from the absorbance at 480 nm after incubation with substrate L-Trp in enzyme or cellular assays conducted in HeLa cells treated with IFN-γ. The half maximal inhibitory concentration (IC_{50}) values were calculated based on percent inhibition compared to that of the vehicle control and are represented in Tables 1–4[5,12,13]

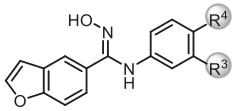
First, the SAR of *N*′-hydroxy-*N*-phenylbenzofuran-5-carboximidamide derivatives was investigated (Table 1). The unsubstituted phenyl derivative **15** showed weak activity with an IC_{50} value of 19 μM in HeLa cells. The 4-chlorophenyl derivative **16** also did not exhibit any activity in either enzyme or cell-based assays. Among the 3-halogenated phenyl derivatives, the 3-fluorophenyl derivative **17** did not improve the inhibitory activity compared to **15**, but the 3-chlorophenyl **18** and 3-bromophenyl **19** derivatives showed potent inhibition with IC_{50} values of 0.69 and 0.44 μM in enzymes and IC_{50} values of 1.5 and 1.1 μM in HeLa cells, respectively. On the other hand, the 3-trifluoromethylphenyl derivative **20** also displayed good inhibition but had a lower inhibition than that of **18** and **19**.

In the di-substituted derivatives, when a 4-fluoro atom was incorporated into **17–20**, thereby producing **21–24**, the activities did not improve, but **23** and **24** still showed good inhibition. The 3-fluoro-4-chlorophenyl **25** and 3,4-dimethylphenyl **26** derivatives were confirmed to be inactive, as expected. Since the incorporation of a polarized bulky group into the 3-position provided good inhibition, other types of bulky groups were further explored. Unfortunately, most of these bulky groups, including alkylcarbonyl (**27**), *N*-alkylamide (**28–30**), alkyloxy (**31**), aryloxy (**32**), and arylalkyl (**33, 34**), were found to be inactive in both enzyme and cell-based assays. Only the 1,1′-biphenyl derivative **35** displayed moderate inhibition.



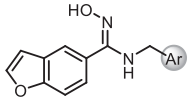
Scheme 2. Synthesis of the 2-alkoxymethyl 5-(*N*-hydroxycarbamimidoyl) benzofuran derivatives *Reagents and conditions*: (a) I_2, 20% NH_4OH, MeOH, r.t., 2 h; (b) propargyl alcohol, $Pd(dppf)Cl_2$, DIPA, CuI, toluene/THF (2:1), 100 °C, 10 h; (c) i) TBSCl, imidazole, DMF, r.t., 30 min; ii) MeI, NaH, DMF, r.t., 1 h; iii) (chloromethyl)cyclopropane, NaH, DMF, r.t., 12 h; (d) DIBAL-H, CH_2Cl_2, −10 °C, 30 min; (e) NEt_3, $NH_2OH \cdot HCl$, CH_2Cl_2, r.t., 3 h; (f) NCS, DMF, 50 °C, 3 h; (g) $R^2(CH_2)_nNH_2$, THF, r.t., 16 h; (h) TBAF, THF, r.t., 1 h.

Table 1
Inhibitory activity of the substituents of the phenyl group.



Compd	R_3	R_4	IDO1 (IC_{50}, μM)	
			Enzyme	HeLa cells
15	H	H	>30	19
16	H	Cl	>30	>30
17	F	H	32.7	20
18	Cl	H	0.69	1.5
19	Br	H	0.44	1.1
20	CF_3	H	1.2	1.9
21	F	F	22.7	14.0
22	Cl	F	5.2	1.8
23	Br	F	1.6	2.6
24	CF_3	F	1.2	2.2
25	F	Cl	>30	10.3
26	Me	Me	>30	>30
27	$COCH_3$	H	>30	>30
28	$CONHCH_2$(c-hexyl)	H	>30	>30
29	$CONHCH_2Ph$	H	>30	>30
30	CO-(4-Me-piperidin-1-yl)	H	>30	>30
31	$O(CH_2)_3CH_3$	H	>30	>30
32	OPh	H	>30	>30
33	CH_2Ph	H	>30	>30
34	CH_2OPh	H	>30	>30
35	Ph	H	11.0	11.9

Table 2
Inhibitory activity of the substituents of the benzyl group.



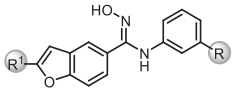
Compd	R	IDO1 (IC_{50}, μM)	
		Enzyme	HeLa cells
36		>30	28.2
37		>30	5.8
38		22	17.6
39		>30	16.2
40		>30	>30
41		>30	>30

SAR analysis indicated that the 4-substitution is detrimental to activity, but the incorporation of a bulky halogen at the 3-position is critical for activity; their potencies seem to be correlated to their corresponding molar refractivity (MR) values (Br = 0.888 > C1 = 0.603 > CF_3 = 0.502 > F = 0.092), which account for the size and polarity of a certain group.

Next, according to the results in Table 1, the one-carbon homologous analogs (**36–41**) of phenyl, 3-chlorophenyl, 3-chloro-4-fluorophenyl, heterocyclic and cyclopropyl rings were examined (Table 2). However, most of them exhibited weak or little activity, indicating that they were less active than the corresponding directly linked derivatives.

Next, the 2-hydroxymethyl (**42–44**), 2-methoxymethyl (**45–47**), 2-(cyclopropylmethoxy)methyl (**48–50**), and 2-phenyl benzofuran (**51–53**) analogs of potent 3-halophenyl derivatives (**17–19**) were explored (Table 3). SAR analysis indicated that they showed a similar pattern as that examined in Table 1, in which the activity increased as the size of the halogen increased. The side chains at the 2-position of benzofuran appeared to be tolerated for activity.

Table 3
Inhibitory activity of the benzofuran C(2)-substituents.



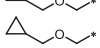
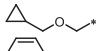
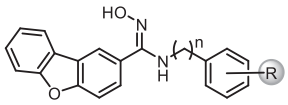
Compd	R^1	R	IDO1 (IC_{50}, μM)	
			Enzyme	HeLa cells
42		F	1.7	2.4
43		Cl	8.0	5.9
44		Br	0.71	1.2
45		F	7.1	6.1
46		Cl	1.0	2.2
47		Br	0.93	2.5
48		F	2.2	8.0
49		Cl	1.5	17.6
50		Br	0.4	3.2
51		F	11	10.6
52		Cl	0.9	10.8
53		Br	0.9	8.6

Table 4
Inhibitory activity of the dibenzofuran scaffold.



Compd	R	n	IDO1 (IC_{50}, μM)	
			Enzyme	HeLa cells
54	3-Cl	0	1.8	7.6
55	3-Br	0	1.3	10.8
56	3-Cl-4-F	0	9.6	22.8
57	3-Br-4-F	0	2.4	16.2
58	3,5-$(OMe)_2$	0	>30	>30
59	3-Cl	1	3.6	29.6

Finally, the dibenzofuran surrogates (**54–59**) of potent inhibitors (**18, 19, 22, 23**) were explored (Table 4). Their activities were similar to those of the 2-phenylbenzofuran derivatives in Table 3 (**52** *vs* **54**, **53** *vs* **55**), indicating that the dibenzofuran ring is the cyclized form of 2-phenylbenzofuran. The SAR showed a similar pattern as that of the benzofuran derivatives.

To investigate the binding mode of the new scaffold for IDO1, docking studies of **19**, the most potent inhibitor, with the IDO1 X-ray crystal structure were performed.[14,15] As shown in Fig. 2, the binding mode of **19** was found to be similar to that of epacadostat.[16] The hydroxyl group of *N*-hydroxyamidine forms a strong dipole-ionic interaction with the heme iron. The 3-bromophenyl group binds deep into the hydrophobic pocket (Pocket A), which is comprised of Tyr126, Val130, Phe164, and Leu234 and forms a π-π stacking interaction with Phe163. In addition, the 3-Br group forms a strong halogen bonding interaction with the sulfur of Cys129. On the other hand, the benzofuran ring occupied the second hydrophobic pocket (Pocket B)

In summary, a series of *N*-hydroxy-*N*′-substituted benzofuran-5-carboximidamide compounds were investigated as IDO1 inhibitors in enzyme and cell-based assays. SAR analysis indicated that in *N*-phenyl substituted derivatives, the position of the substituents at the phenyl

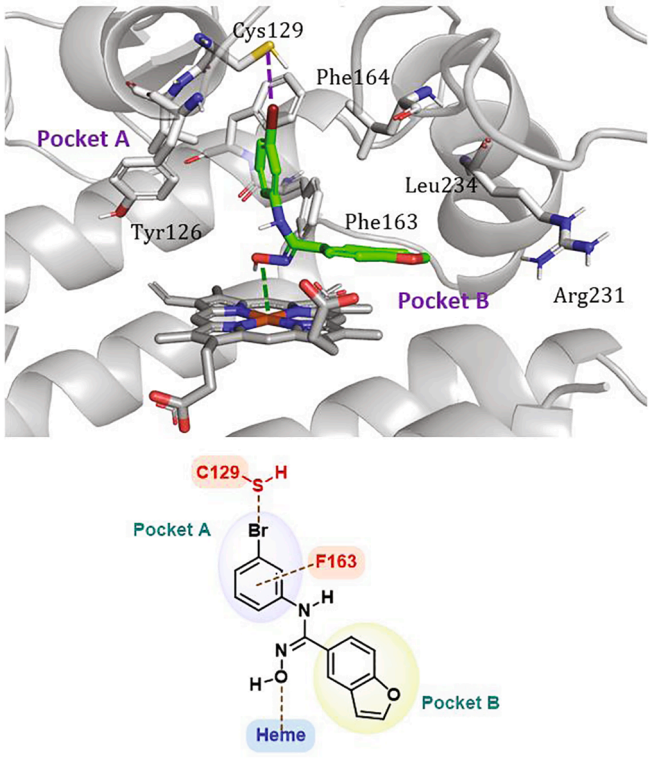


Fig. 2. Predicted binding mode of **19** in IDO1. The key interacting residues are marked and displayed as capped-stick representations with carbon atoms in gray. Halogen bonding is drawn as a purple dashed line, and bonding with heme is displayed as a green dashed line. The bottom panel shows the 2D interactions.

ring exerted an influence on potency, with the 3-position being the most potent. Among the 3-substituents, the halogens provided a high potency with increasing size (Br > Cl > F), probably due to their capability to form halogen bonds, but other types of substituents did not produce any beneficial effects compared to those of the unsubstituted phenyl derivatives. The one-carbon homologous and dibenzofuran analogs were found to be inactive. Molecular modeling of the most potent **19** in the X-ray crystal structure of IDO1 indicated that the dipole-ionic interaction of *N*-hydroxyl group with the heme iron, the halogen bonding of the bromo atom with the sulfur of cysteine and the two hydrophobic interactions were the key interactions for the high potency of **19**.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

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