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Discovery of novel hydroxyamidine derivatives as indoleamine 2,3-dioxygenase 1 inhibitors with *in vivo* anti-tumor efficacy

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ABSTRACT

Indoleamine 2,3-dioxygenase 1 (IDO1) is closely associated with immune escape in many tumor tissues, and is considered to be a valuable therapeutic target in cancer immunotherapy. In this study, the modification of amino sidechain was performed with the hydroxyamidine core kept intact to optimize lead compound Epacadostat. 19 new compounds with hydrazide, thietane or sulfonamide moiety as polar capping group in sidechain were prepared and their IDO1 inhibitory activities were evaluated. Sulfonamide **3a** showed potent IDO1 inhibition in both enzymatic and cellular assays with the IC_{50} value of 71 nM and EC_{50} value of 11 nM, respectively. Furthermore, *in vivo* Lewis lung cancer (LLC) allograft studies of **3a** indicated that it handicapped the tumor growth with similar efficacy to Epacadostat. Molecular docking demonstrated that the change of polar capping group affords influence on the orientation of amino ethylene side chain and forms new hydrogen bonding.

The indoleamine 2,3-dioxygenase 1 (IDO1), an extrahepatic cytosolic heme-containing dioxygenase, is the rate-limiting enzyme on the tryptophan kynurenine pathway.[1,2] Through this pathway, the tryptophan is oxidized to *N*-acetyl kynurenine, which is subsequently converted to kynurenine, 3-hydroxykunurenine, quinolinic acid and other metabolites.[3] The overexpression of IDO1 in cancer cells leads to tryptophan depletion and kynurenine pathway metabolites accumulation. Tryptophan depletion induces effector T cells incompetence and apoptosis through general control nonderepressible 2 (GCN2) activation[4] and mechanistic target of rapamycin kinase (mTOR) inhibition[5] pathway. Kynurenine pathway metabolites accumulation stimulates T cell differentiate to regulatory T cells (Tregs) as a consequence of binding to the aryl hydrocarbon receptor (AHR).[6] Through above mentioned approach, cancer cells evade immune surveillance in many tumor tissues.[7–12] Therefore, inhibition of IDO1 has become an attractive approach in cancer immunotherapy.

Tremendous efforts have been made to discover potent small-molecule IDO1 inhibitors.[13–15] Several IDO1 inhibitors with elucidated molecular structure have entered various stages of clinical trials, including Indoximod,[16] Epacadostat,[17] Navoximod,[18] PF-0684003[19] and Linrodostat.[20] (Fig. 1) Among them, Epacadostat had increased response when combined with nivolumab or pembrolizumab in phase I/II trials.[17,21] Although the enhancement of Epacadostat was not observed during combination with pembrolizumab in the recent phase III ECHO 301 trial,[22] which may be related to insufficient drug exposure or mismatched drug combination strategy,[23,24] IDO1 related therapy is still a promising area.

According to the co-crystal structure of IDO1 bound to Epacadostat,[25] the inhibitor forms vital coordination with heme iron via the oxygen atom of the hydroxyamidine group. The halogenated phenyl group occupies pocket A and was surrounded by a group of hydrophobic residues. The Br and F atoms offer a favorable fluorine–sulfur contact with C129 in the *N*-terminal domain. The central furazan group is stabilized by F163, L234, and F226. Moreover, the sulfamoylamino ethylene sidechain pointing into pocket B, with the sulfamoylamino group stabilized by R231 through hydrogen bonding. The halogenated phenyl region and coordinating group of Epacadostat have been comprehensively optimized in Incyte's previous reports.[26,27] However, only a few types of polar sidechain were investigated,[28–30] despite the fact that pocket B is not fully occupied and variable side part with polar group may be tolerated.

In this study, lead optimization of Epacadostat was performed focusing on the modification of amino sidechain with the hydroxyamidine substituted furazan moiety kept intact to keep the two important intramolecular hydrogen bonds that forces amidine C=N double bond to hold a cis conformation, which is critical for the

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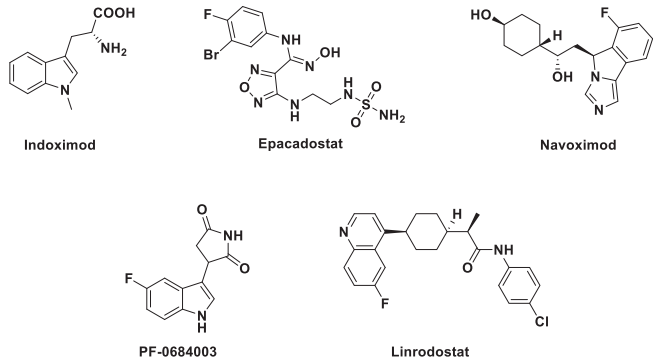


Fig. 1. Structures of representative IDO1 inhibitors in clinical stage.

inhibitory activity.[27] Hydrazide derivative was initially constructed as removal of ethyl linker and directly attaching the sulfamoylamine component with amino group in sidechain. Moreover, thietane derivatives with sulfoxide, sulphone, *N*-cyano sulfilimine or sulfoximine moiety were designed to reduce the fixability of sidechain and fix the orientation of polar capping group. Due to irrelevant interaction between the inner NH in the Epacadostat sulfamoylamine group and pocket B of IDO1,[25] sulfonamide and its substituted analogues were also explored. Herein, the syntheses and SAR of novel hydroxyamidine derivatives (Fig. 2) as new IDO1 inhibitors are communicated.

The synthetic methods of all desired compounds were illustrated in Schemes 1–4. As shown in Scheme 1, the syntheses of **1a–c** started from 3-(4-amino-1,2,5-oxadiazol-3-yl)-4-(3-bromo-4-fluorophenyl)-1,2,4-oxadiazol-5(4*H*)-one (**6**) which was prepared according to previously reported methods.[27] Oxidation of **6** with hydrogen peroxide afforded 4-nitro-1,2,5-oxadiazole derivative (**7**). Upon hydrazide substitution, key intermediate 4-hydrazinyl-1,2,5-oxadiazole **8** was generated in 63% yield. After mesylation and further hydrolyzation, the target compound methanesulfonyl hydrazide (**1a**) was obtained. It was noted that the aminosulfonyl moiety in **1c** was unstable under basic deprotection condition and can't be constructed by the similar route. To obtain **1c**, aminosulfonyl group was directly introduced into deprotected hydrazide derivative **1b**, which was prepared from the hydrolysis of **8** under basic condition.

To fix the orientation of polar capping group, thietane analogues (**2a–d**) were synthesized as described in Scheme 2. The substitution of nitro compound **7** with thietan-3-amine generated thietane derivative (**10**). After oxidation with IBX or *m*-CPBA and hydrolysis with NaOH, the target compounds **2a** and **2c** were obtained, respectively. For the preparation of target compound containing *N*-cyano sulfilimine (**2b**) or sulfoximine (**2d**), **10** was oxidized with (diacetoxyiodo)benzene in the presence of ammonium carbamate or cyanamide, and directly hydrolyzed without purification.

Sulfonamide derivative (**3a**) as removing inner nitrogen atom of sulfamoylamine group in Epacadostat and its substituted analogues (**3b–i**) were constructed according to Scheme 3. The above nitro compound **7** was substituted with taurine to obtain sodium sulfonate (**15**) with ethylene linker in sidechain. After activation with oxalyl chloride, substitution with varied amines (for **3a–g**), hydrazine (for **3h**) or hydroxylamine (for **3i**) and further hydrolyzation with 2 N NaOH in MeOH, the desired target sulfonamide derivatives **3a–i** were prepared. Installation of the side chain with propylene linker was also achieved with similar method to afford sulfonamide **4a** and **4b**. Moreover, linker between oxadiazole core and polar capping group was changed from

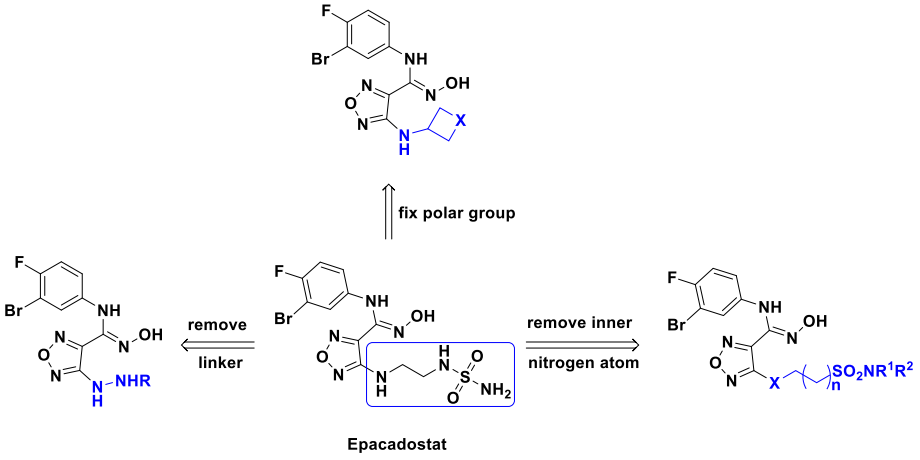
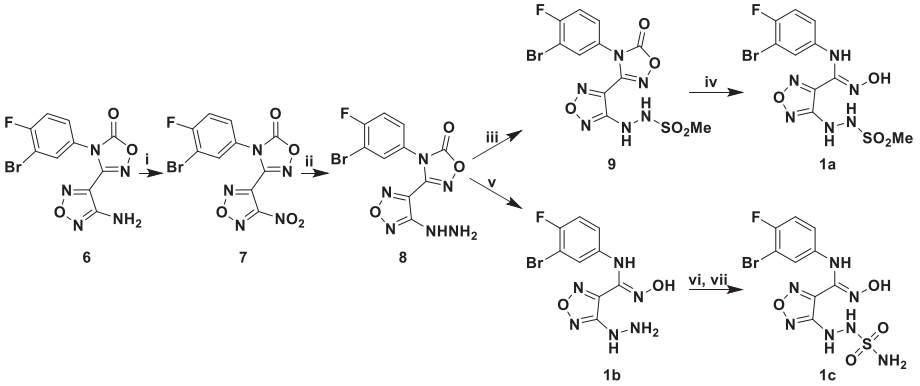


Fig. 2. Design concept of novel Epacadostat analogues.



Scheme 1. Synthesis of the compounds **1a–c**. Reagents and conditions: (i) TFA, H_2O_2, 80 °C, 70%; (ii) N_2H_4, DMF, rt, 63%; (iii) MsCl, NEt_3, rt; (iv) NaOH, MeOH, rt, 32%; (v) NaOH, MeOH, rt, 60%; (vi) $ClSO_2NCO$, *t*-BuOH, NEt_3; (vii) TFA, DCM, 82%.

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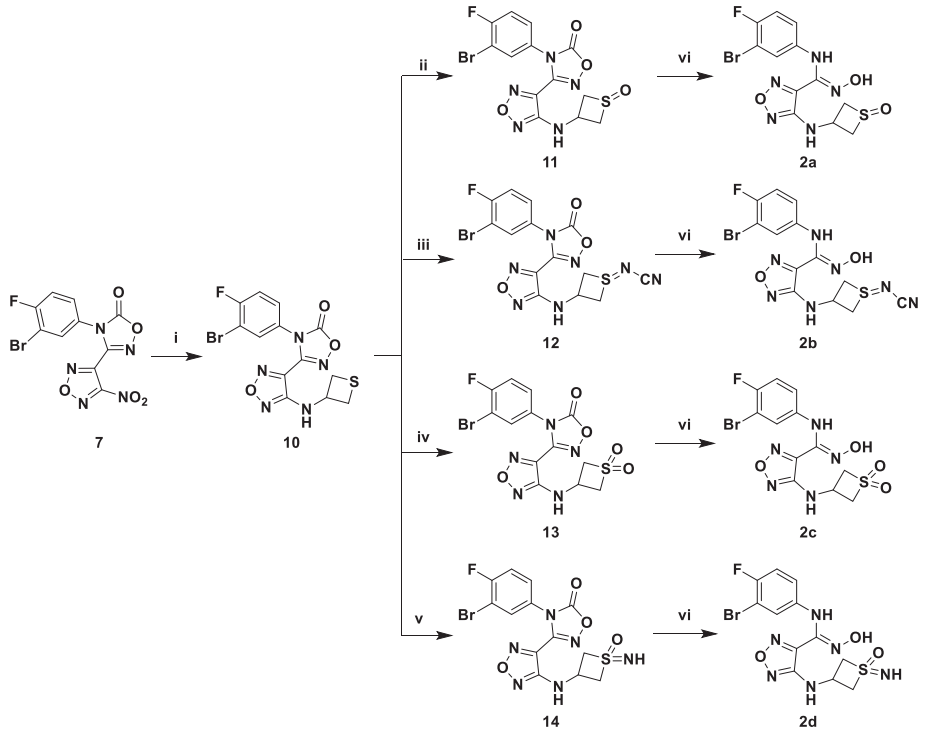
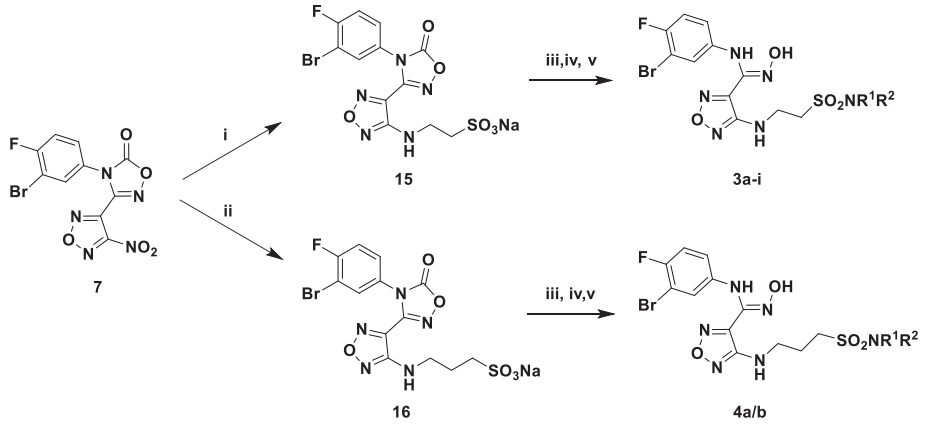


Scheme 2. Synthesis of the compounds **2a–d**. Reagents and conditions: (i) thietan-3-amine, NEt_3, THF, 63%; (ii) IBX, benzyltriethylammonium chloride, DCM, H_2O; (iii) NH_2CN, (diacetoxyiodo)benzene, CH_3CN; (iv) *m*-CPBA, DCM; (v) ammonium carbamate, (diacetoxyiodo)benzene, MeOH; (vi) NaOH, MeOH, 37–73%.

ethylamine to ethoxyamine and the compound **5** was prepared with similar method to **3a–i** (Scheme 4).

With all designed compounds in hand, we first tested their enzyme-based IDO1 inhibition activity according to the reported procedure,[32,33] and lead compound Epacadostat was used as positive control. As shown in Table 1, replacement of amino sidechain with hydrazide, thietane or sulfonamide played significant influences on IDO1 inhibition. Six of the synthesized compounds exhibit the potent inhibition of IDO1-mediated conversion of tryptophan to kynurenine with IC_{50} values less than 100 nM. The ethylene linker in sidechain is critical for the activities. Removal the ethylene group is detrimental to inhibitory activity (**1c** vs Epacadostat), with IC_{50} value of 218 nM. Replacing external amino group with methyl further results 3-fold decrease of inhibitory activity (**1a** vs **1c**). The hydrazine intermediate **1b** showed 2-fold higher potency comparing to the target molecule **1c**, but still lower than Epacadostat. These results indicated that hydrazide and its derivatives maybe weaken the interaction between the polar sidechain and pocket B.

Fixing the orientation of polar capping group with thietane was tolerated, with IC_{50} values in the range of 69–212 nM. Thietane derivative containing sulphoxide moiety lead to 3-fold decrease of activity (**2a** vs Epacadostat). But replacement of sulphoxide with *N*-cyano sulfilimine (**2b**) exhibited comparable inhibitory potency (IC_{50} = 69 nM) to Epacadostat. Thietane derivative with sulfone or sulfimide group resulted in 1.5 to 3-fold loss in potency (**2c**/**2d** vs Epacadostat), with IC_{50} value of 99 or 208 nM, respectively. It might suggest that sulfone moiety afforded better H-bonding over sulfimide or sulphoxide (**2c** vs **2d**/**2a**).



a: R^1 = H , R^2 = H; b: R^1 = Me , R^2 = Me; c: NR^1R^2 = azetidine; d: R^1 = H , R^2 = Et; e: R^1 = H , R^2 = CH_2CF_3
f: R^1 = H , R^2 = prop-2-yn-1-yl; g: R^1 = H , R^2 = cyclopropylmethyl; h: R^1 = H , R^2 = NH_2; i: R^1 = H , R^2 = OH

Scheme 3. Synthesis of the compounds **3a–i** and **4a–b**. Reagents and conditions: (i) 2-aminoethane-1-sulfonic acid, NaOH, H_2O, DMF, 79%; (ii) 3-aminopropane-1-sulfonic acid, NaOH, H_2O, DMF, 37%; (iii) $(COCl)_2$, DMF, THF; (iv) NR^1R^2, NEt_3, THF; (v) NaOH, MeOH, H_2O, 19–69%.

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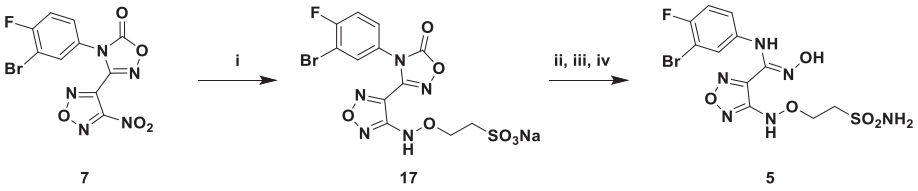
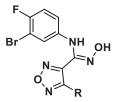
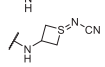
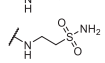
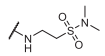
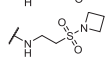
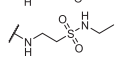
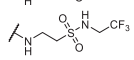
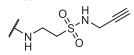
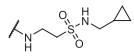
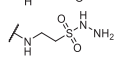
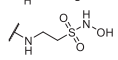
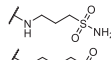
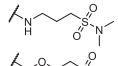
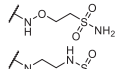
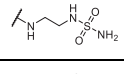


Scheme 4. Synthesis of the compound **5**. Reagents and conditions: (i) 2-(aminooxy)ethane-1-sulfonic acid,[31] NaOH, H_2O, DMF, 58%; (ii) $(COCl)_2$, DMF, THF; (iii) NH_3, THF; (iv) NaOH, MeOH, H_2O, 31%.

To our delight, the sulfonamide derivative **3a** which removing inner NH in Epacadostat sulfamoylamine group displayed similar IDO1 inhibition activity (IC_{50} = 71 nM) comparing to the lead compound. The hydrogen atom in sulfonamide was further replaced with a variety of hydrophobic or hydrophilic substituents. When small hydrophobic substitution ethyl or trifluoroethyl were introduced in sulfonamide moiety, enzymatic activity slightly decreased (**3d**/**3e** vs **3a**), with IC_{50} values of 95 nM and 140 nM, respectively. And introduction of large substitution such as prop-2-yn-1-yl and cyclopropylmethyl, further decreased enzymatic activity were observed (**3f**/**3g** vs **3d**/**3e**). These data suggested that hydrophobic substitution, especially large steric substitution is not conducive for the sidechain of the compounds extending into the pocket B. *N*,*N*-Di-substituted sulfonamide derivatives leaded to 1.5–2.7 fold drop in inhibitory activity (**3b**/**3c** vs **3a**). Hydrophilic hydroxyl (**3 h**) and amino (**3i**) substitution also exhibited almost 2-fold less potency than **3a**. Moreover, a change from ethylene linker to propylene group resulted in slightly loss in potency (**4a** vs **3a**), with IC_{50} value of 126 nM. Alternatively, we changed the linker between oxadiazole core and polar capping group from ethylamine to ethoxyamine and the obtained compound **5** showed slightly reduced activity (IC_{50} = 87 nM).

Table 1
IDO1 inhibitory activity of **1a–c, 2a–d, 3a–i, 4a–b** and **5.**



Comp.	R	IDO1 IC_{50} (nM)[a]	HeLa EC_{50} (nM)[a]
1a		609 ± 45	> 800
1b		108 ± 16	556 ± 44
1c		218 ± 12	101 ± 13
2a		212 ± 47	79 ± 16
2b		69 ± 7	73 ± 16
2c		99 ± 6	59 ± 7
2d		208 ± 10	53 ± 11
3a		71 ± 6	11 ± 1
3b		105 ± 1	17 ± 2
3c		191 ± 42	143 ± 1
3d		95 ± 11	41 ± 4
3e		140 ± 15	268 ± 18
3f		218 ± 10	33 ± 3
3g		312 ± 11	268 ± 18
3h		135 ± 11	> 800
3i		126 ± 14	> 800
4a		126 ± 4	15 ± 1
4b		97 ± 7	20 ± 1
5		87 ± 3	27 ± 1
Epacadostat		66 ± 9	8 ± 1

[a] IC_{50}/EC_{50} values are the mean of three independent assays.

The obtained compounds were also evaluated for their inhibitory activity for tryptophan depletion and simultaneous kynurenine production in HeLa cell with stimulation of IFN-γ.[32–33] As shown in Table 1, most of compounds inhibit the IDO1-mediated conversion of tryptophan to kynurenine in cell-based assay with EC_{50} values less than 200 nM. Hydrazine derivatives **1a–c** showed decreased inhibitory effect compared to Epacadostat, with EC_{50} values lager than 100 nM. Thietane derivatives **2a–d** exhibited better inhibitory activity than **1a–c**, but still lower than Epacadostat. Fortunately, non-substituted sulfonamide **3a** displayed potent IDO1 inhibitory activity in cell (EC_{50} = 11 nM), which is comparable with Epacadostat. Further hydrophobic mono- or di-substitution in sulfonamide group all showed reduced inhibitory effect (**3b–g** vs **3a**). And hydrophilic substitution (**3 h** and **3i**) lead to totally loss of inhibitory activity. Moreover, increasing chain length from ethylene linker to propylene group resulted in slightly loss in potency (**4a**/**4b** vs **3a**), with EC_{50} values 15 nM and 20 nM, respectively. Compound **5**, which changing linker from ethylamine to ethoxyamine, lead to 2-fold drop of inhibitory potency (EC_{50} = 27 nM) compared with **3a**. In general, most compounds showed a good correlation between enzymatic and cellular assays.

Encouraged by the potent inhibitory activity of **3a** on enzyme-based and cell-based assay, we further investigated the anti-tumor efficacy *in vivo*. The LLC (Lewis lung cancer) allograft model in immunocompetent mice was adopted[34] and Epacadostat was used as positive control. When the tumors diameter roughly reached 5 mm, the tested compound **3a** or Epacadostat was administered with oral dosing (100 mg/kg) twice a day for 14 days. As shown in Fig. 3, compared to the vehicle control, both compound **3a** and Epacadostat obviously inhibited the tumor growth. The sulfonamide derivative **3a** proved to show similar efficacy compared to Epacadostat, which is consistent with their tendency of cellar IDO1 inhibitory. In addition, mice were well tolerated to **3a** with no significant body weight loss until the end of assay (Fig. S1).

To elucidate the binding model of **3a** with IDO1, molecular docking was preformed based on the crystal structure of IDO1 complexed with Epacadostat (PDB code: 5WN8)[25] using Schrodinger (Maestro suite). As shown in Fig. 4, **3a** (orange carbons) bind in almost the same location as that of Epacadostat (cyan carbons). The phenyl group of occupies the pocket A and was surrounded by hydrophobic residues with fluorine-sulfur contact. The oxygen of hydroxyamidine coordinates to the ferrous iron of the heme. However, removing inner amino of sidechain changes the orientation of amino ethylene side chain. The sidechain of **3a** forms two hydrogen bonds between sulfonamide nitrogen atom and L234/G262, nevertheless sulfonamide oxygen atom in Epacadostat interacts with R231 and forms one hydrogen bond. All these

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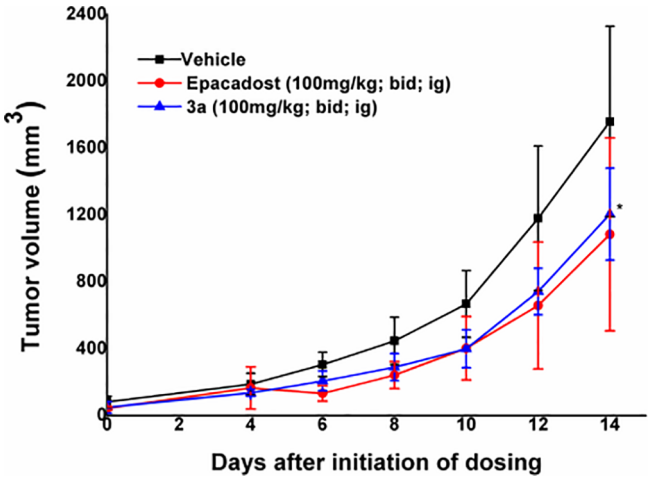

Fig. 3. Tumor growth curve upon treatment with **3a**, Epacadostat or vehicle in C57BL/6 mice bearing LLC allografts. Vehicle: 0.5% CMC suspension. Tumor volume for indicated days was showed as means ± SEM (n = 6). (*) *P* less than 0.05 compared with the vehicle group.

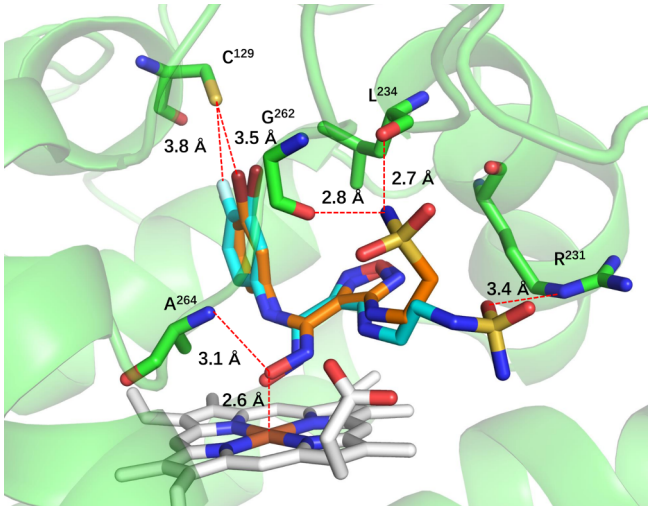

Fig. 4. Predicted binding modes for compound **3a** (orange carbons) with the active site of the human IDO1 enzyme in comparison with Epacadostat (cyan carbons).

computational predictions explain the comparable potency of **3a** with Epacadostat.

In summary, a series of Epacadostat analogues with hydrazide, thietane and sulfonamide as side part were designed and constructed as IDO1 inhibitors. The *in vitro* IDO1 inhibition assay illustrated that sulfonamide (**3a**) is the most suitable type of sidechain with the enzymatic IC_{50} value of 71 nM and cellular EC_{50} value of 11 nM, respectively. Efforts were made to optimize substitution in sulfonyl amide. Unfortunately, no matter hydrophobic or hydrophilic substitution leaded to a drop of inhibition efficacy. *In vivo* antitumor studies of **3a** indicated similar efficacy with Epacadostat. Moreover, molecular docking demonstrated that the change of polar capping group affords influence on the orientation of amino ethylene side chain and forms the new hydrogen bonding. The SAR relationship of Epacadostat analogues with varied sidechain provides the new information for drug design of novel IDO inhibitors.

Acknowledgment

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmcl.2020.127038.

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