

## Regular Article

## A Novel High Throughput Virtual Screening Protocol to Discover New Indoleamine 2,3-Dioxygenase 1 (IDO1) Inhibitors

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Indoleamine 2,3-dioxygenase 1 (IDO1) plays an important role in the immune escape of tumors and has emerged as a promising target for cancer immunotherapy. Despite its potential in immuno-oncology, very few chemotypes have been reported to date. Here, we designed a novel high throughput virtual screening (HTVS) cascade protocol, combining both pharmacophore modeling and molecular docking and it was employed to query commercially available compounds to identify novel inhibitors. Among the 23 compounds selected for the *in vitro* IDO1 inhibitory activity assay, five compounds exhibit greater than 20% inhibition at a test concentration of 10  $\mu\text{M}$ , with two compounds having an  $\text{IC}_{50}$  value of 23.8 and 8.8  $\mu\text{M}$ , respectively. The novel scaffold together with a ligand efficiency of 0.28 kcal/mol per heavy atom makes both compounds as suitable starting points for future chemistry elaboration. Our HTVS protocol was validated and could be employed in discovery of IDO1 inhibitors.

**Key words** indoleamine 2,3-dioxygenase 1; inhibitor; immuno-oncology; virtual screening

Tumor escape is a hallmark of cancer, which brings many difficulties and troubles in the cancer therapy.<sup>1</sup> Indoleamine 2,3-dioxygenase 1 (IDO1) has been proved to be closely associated with the immune escape of cancer.<sup>2,3</sup> IDO1 is a monomeric 45 kDa extrahepatic heme-containing dioxygenase. It is the rate-limiting enzyme in the kynurenine pathway that plays an essential role in tumor escape.<sup>4</sup> While IDO1 is silent in most tissues, it is highly up-regulated to acquire peripheral tolerance, e.g., tolerance to placenta.<sup>5</sup> Many studies demonstrate that the abnormal high level of IDO1 is found in patients with quite a few carcinomas, such as ovarian carcinoma, hepatocellular carcinoma, invasive cervical carcinoma, metastatic melanoma, non-small cell lung carcinoma and metastatic prostate carcinoma.<sup>6</sup> Apart from cancer, IDO1 has been connected to common diseases such as Alzheimer's disease and melancholia.<sup>7</sup>

Design of selective and highly active IDO1 inhibitors has attracted increasing interest. The first-generation inhibitors were tryptophan and indole derivatives, with 1-methyl-L-tryptophan (1-MT) having an  $\text{IC}_{50}$  value of 380  $\mu\text{M}$  identified in 1991.<sup>8,9</sup> The 1-MT has been used as a tool compound in many studies involving IDO1 and immunotherapy. However, little progress has been made on the tryptophan and indole derivatives, due to their poor cellular activity.<sup>3</sup> IDO inhibitors from natural sources were identified to show moderate inhibitory activities, such as Norharman Tryptanthrin, a natural product from the Chinese medicinal plants. Its derivatives can reach nM inhibitory activity on IDO1, and suppress tumor growth on Lewis lung cancer (LLC) tumor-bearing mice.<sup>10</sup> The most promising IDO1 inhibitors are Epacadostat<sup>11</sup> and NLG919, both advanced in clinical trials (Fig. 1).

Despite the intensive interest on IDO1 inhibitors, only few chemotypes have been reported to date.<sup>12</sup> Although there were many basic studies on the IDO1 and IDO1 inhibitors, the studies on high throughput virtual screening (HTVS) for IDO1 inhibitors remained very few and most of them were done before 2012.<sup>13,14</sup> Here, we designed an effective HTVS proto-

col combining both pharmacophore modeling and molecular docking with the help of many basic studies after 2012 such as structure–activity relationship (SAR), binding modes and fresh released crystal structure. Successfully, we report on the discovery of two novel IDO1 inhibitors by our HTVS protocol (Fig. 2).

## Experimental

**Processing and Preparation of Protein** The X-ray crystallographic structural details of the indoleamine 2, 3-dioxygenase 1 in complex with NLG919 analogue with a resolution factor of 2.21 Å (PDB code: 5EK3) was retrieved from RCSB Protein Data Bank (PDB).<sup>15</sup> The 5EK3.pdb file was imported to Discovery Studio 3.0 Client (DS 3.0) (Accelrys Inc., San Diego, CA, U.S.A.) for the process to ensure that the retrieved structure was reliable and qualitatively considerable for further *in silico* studies. The missing hydrogen atoms were

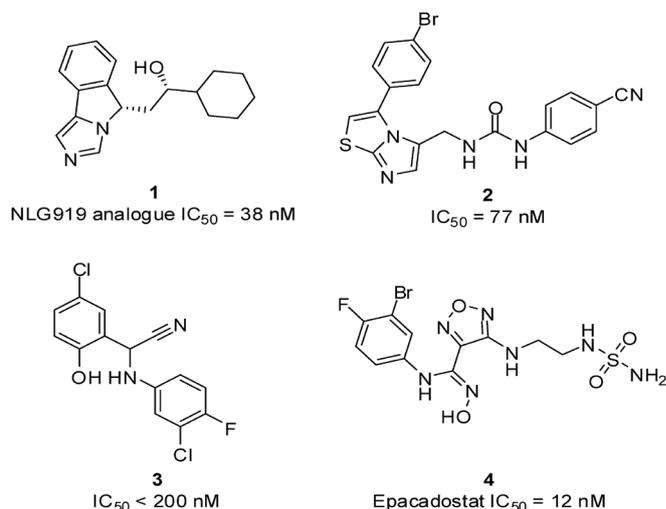


Fig. 1. Known IDO1 Inhibitors

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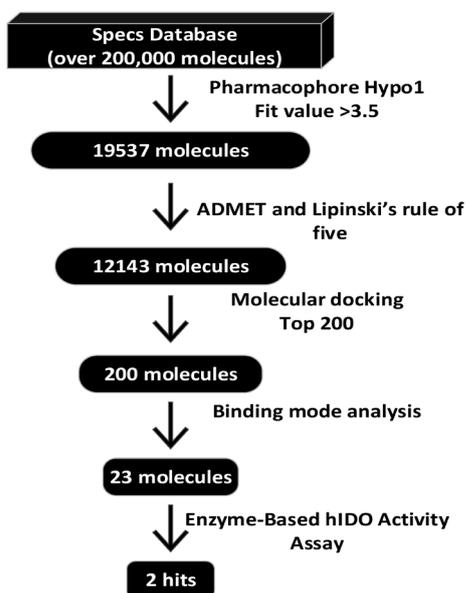


Fig. 2. Flowchart of the Virtual Screening Cascade Protocol

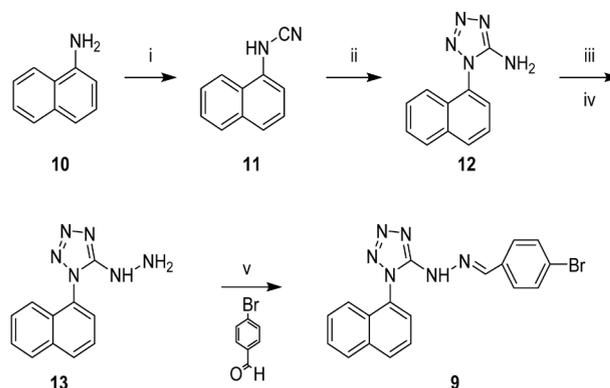
corrected and the water molecules were removed. We also built loops and assigned suitable protonation states at pH 7.4 and minimized the energy of the structure and so on.

**Preparation of Ligands** Compounds in Specs database were selected in the *in silico* study, since it is open-accessible and the compounds could be purchased for further *in vitro* experiment easily. A slice of reported active ligands were selected for pharmacophore modeling and investigating their interactions with the IDO1 protein. All the ligands were prepared by using DS3.0 Client and were in a ready-to-dock 3D format.

**Generation of Pharmacophore** Pharmacophore modeling is an effective approach to discover original and novel scaffolds for a particular target. There are several ways to generate pharmacophores automatically in DS3.0, such as from bioactive conformation, from a receptor–ligand complex, from a set of ligands and so on. In view of the huge distinct of the reported active scaffolds, pharmacophores were generated by four structurally distinct and potent IDO1 inhibitors<sup>11,15,16</sup> (Fig. 1) and modified to search database. Due to the short bonding distances between the ligands and the neighboring residues and the heme of the IDO1 active site, excluded volumes were created manually to avoid potential collisions and eliminate unsatisfactory compounds.

**Database Search Based on Pharmacophore Model** The prepared ligands in specs database were used in the compound search. It has been shown that direct coordination to the heme iron is vital to the IDO1 inhibitory activity, so hydrogen bond acceptor was set as a required feature. The other parameters in protocol were set as default. Molecules which fit value >3.5 were retrieved as candidate compounds.

**ADMET Prediction and Lipinski's Rule of Five** The failure of many drug candidates in clinical trials can be attributed to poor ADMET (absorption, distribution, metabolism, excretion, toxicity) properties. Computer-aided ADMET prediction allows us to eliminate compounds with unfavorable ADMET properties early to avoid expensive development costs. DS 3.0 ADMET was employed in our study. Compounds with the value of ADMET\_Solubility=0,



Reagents and conditions: (i) NaHCO<sub>3</sub>, BrCN, Et<sub>2</sub>O, r.t., 36 h, 56%; (ii) NH<sub>4</sub>Cl, NaN<sub>3</sub>, DMF, 90°C, 5 h, 83%; (iii) HCl, NaNO<sub>2</sub>, H<sub>2</sub>O, 4°C, 0.5 h; (iv) Zn, HOAc, H<sub>2</sub>O, 10°C, 2 h, 24% (2 steps); (v) EtOH, r.t., 1 h, 88%.

Chart 1. Synthetic Route of Compound 9

ADMET\_BBB\_Level=5 or ADMET\_Absorption\_Level=3 would be eliminated by ligand filter. Only the compounds pass Lipinski's Rule of five would be retained for the following investigation.

**Molecular Docking** CDOCKER module of DS 3.0 was applied for docking studies. Protein and ligands were prepared as described above. A 15 Å radius sphere was defined around the bounded ligand to compose the active site of the IDO1 protein.

**Enzyme-Based IDO1 Inhibition Assay** The enzymatic inhibition assays were performed as described by Su-ying Wu with some minor modifications.<sup>15</sup> Briefly, 50 nM of IDO1 and required concentration of compounds were added to 100 μL of standard assay medium, which includes 50 mM potassium phosphate buffer (pH 6.5), 10 mM ascorbic acid (neutralized with 1 M NaOH solution), 10 μM methylene blue, 100 g/mL catalase, 200 μM L-tryptophan in 96-well black plate. The prepared reaction was incubated at 37°C for 30 min. To stop, 50 μL 30% (w/v) trichloroacetic acid was added to the reaction. The mixture was incubated for 30 min at 52°C, and then centrifuged at 12000×g at room temperature for 10 min. One hundred microliters supernatant was mixed with 100 μL Ehrlich's reagent. The amount of kynurenine production from reaction was measured by the emission of fluorescence at 480 nm using a Victor 2 multi-label reader (PerkinElmer, Inc.). The inhibition percentage was determined as [100−(A/B) 100]/100, where A was the IDO1 activity with the test compounds added to the protein and B was the absence of the test compounds.

**Chemistry** Compound 9 was resynthesized according to Chart 1.

## Results and Discussion

The four structurally distinct and potent IDO1 inhibitors (Fig. 1) were used to generate a 3D pharmacophore model, which briefly consists of two hydrophobic regions, one hydrogen bond acceptor, and one hydrogen bond donor (Fig. 3). In addition, excluded volumes were added manually to avoid steric collisions, upon inspection of the crystallographic structure of IDO1 in complex with an NLG919 analogue (PDB code: 5EK3) (Fig. 3E). It has been shown that direct coordination to the heme iron is vital to the IDO1 inhibitory activity, so hydrogen bond acceptor was set as a required feature when we screened database. The original Specs compounds library

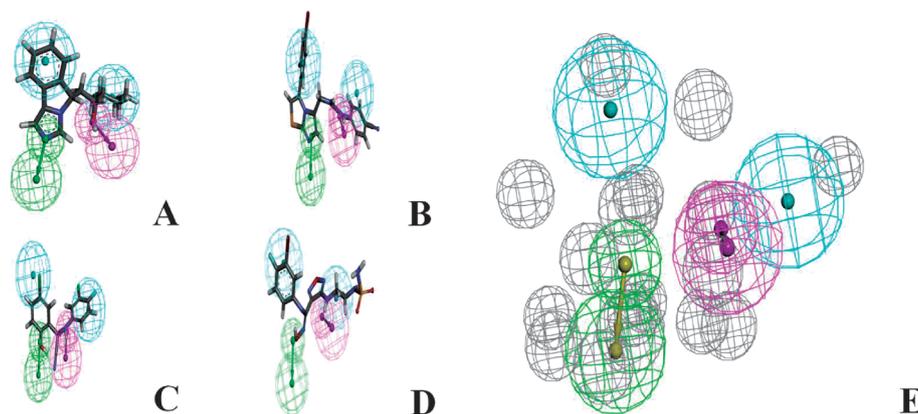


Fig. 3. The Chemical Features of the Best Pharmacophore Hypothesis Aligned with (A) Compound 1, (B) Compound 2, (C) Compound 3, (D) Compound 4 with Hydrogen Acceptor Indicated as Green, Hydrogen Acceptor Indicated as Pink and Hydrophobic Areas Indicated as Cyan, (E) Hypothesis with Excluded Volumes Indicated as Grey

Table 1. Activity of the Five Novel IDO1 Inhibitors Identified by Virtual Screening

Hit	Structure	%Inhibition at 10 $\mu\text{M}$	$\text{IC}_{50}$ ( $\mu\text{M}$ )
5		24.4	N.D.
6		21.4	N.D.
7		28.4	N.D.
8		35.9	23.8
9		53.3	8.8

contains over 200000 molecules. The generated 3D pharmacophore model efficiently reduces the number to 19537. A recent analysis of the attrition of drug candidates from four major pharmaceutical companies indicates a link between the physicochemical properties of compounds and clinical failure due to safety issues.<sup>17)</sup> As such, compounds with violation of Lipinski's Rule of 5 were removed. Moreover, compounds with poor predicted ADMET properties were further removed. The 12143 passed compounds were then subject to molecular docking by CDocker. The top 200 compounds ranked by the docking energies were visually inspected for the detailed molecular interactions. As mentioned early on, only compounds capable of forming a direct coordination with the heme in the active site were kept. Further accounting for structural novelty and diversity, finally 23 compounds were

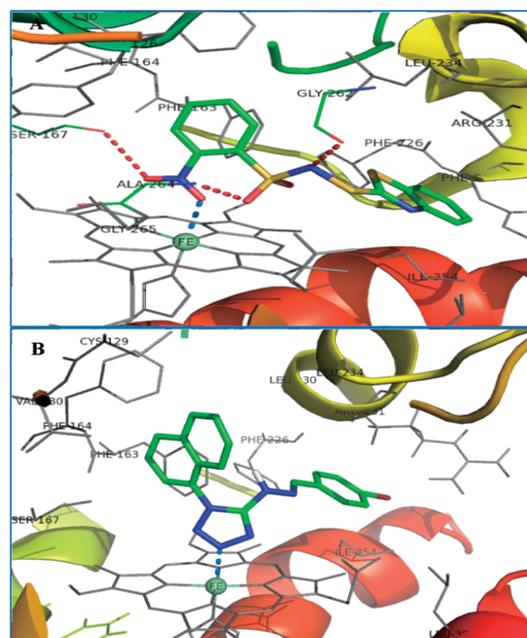


Fig. 4. Predicted Binding Modes of Compounds 8 (A) and 9 (B) in the Active Site of IDO1

The coordination to heme iron was highlighted by a blue dash line and hydrogen bonds were shown in red dash lines.

selected for *in vitro* IDO1 inhibitory assay.

The enzymatic inhibition assay was performed as previously reported<sup>15)</sup> with minor modifications. Among the 23 compounds tested at a single concentration of 10  $\mu\text{M}$ , five exhibited greater than 20% inhibition (Table 1, Table S1). The  $\text{IC}_{50}$  values of compounds 8 and 9 were further determined by a 7-dose response measurement. Notably, compound 9 showed an  $\text{IC}_{50}$  value of 8.8  $\mu\text{M}$  with a ligand efficiency of 0.28 kcal/mol per heavy atom.

The interaction of both compounds 8 and 9 with IDO1 is characterized by the direct coordination to the Heme iron, a typical feature among IDO1 inhibitors (Fig. 4). Compound 8 was the first IDO1 inhibitors to bind the heme iron with the nitrate directly. Compound 8 is further hydrogen bonded to Ser167 side chain by its nitro as an acceptor, to Ala264 backbone amide by its sulfonamide oxygen as an acceptor, and

to Gly262 backbone oxygen by its sulfonamide nitrogen as a donor. The phenyl ring attached with the nitro group establishes a  $\pi$ - $\pi$  stacking interaction with Phe163 side chain (Fig. 4A). The IDO1 inhibitors with tetrazole group like compound **9** remain very few, while it is the first time to discover a compound with the naphthalene group bound to tetrazole. The naphthalene of compound **9** forms not only establishes a  $\pi$ - $\pi$  stacking interaction with Phe163 side chain, but also it forms hydrophobic interactions with the surrounding hydrophobic residues (Fig. 4B).

Compounds from commercial sources may contain considerable impurities or have degraded over time.<sup>18)</sup> To ensure the compound identity, compound **9** was resynthesized according to Chart 1, in addition to synthetic feasibility check for future chemistry elaboration. The resynthesized compound **9** has the same  $IC_{50}$  value of  $8.8\ \mu M$  as the purchased one. Therefore, the low-micromolar inhibitory activity of compound **9** is not an artifact due to impurities.

### Conclusion

In summary, the subject of this manuscript is to design a high throughput virtual screening cascade protocol combining both pharmacophore modeling and molecular docking to search for potential IDO1 inhibitors. We have designed an effective HTVS protocol with the help of many basic studies after 2012 such as SAR, binding modes and fresh released crystal structures. We successfully identify two novel IDO1 inhibitors of moderate activity, and 5 out of 23 compounds displayed an inhibitory potency  $>20\%$  at a concentration of  $10\ \mu M$  meaning that our protocol was the most effective virtual screening protocol for IDO1 inhibitors up to date. Given their structural novelty and moderate ligand efficiency, compounds **8** and **9** may serve as interesting starting points for future chemistry elaboration in the rising field of immuno-oncology. Although the compound **9** is similar to a previously discovered IDO1 inhibitor NRB04258 with  $IC_{50}$  value of  $54\ \mu M$  by Röhrig *et al.*,<sup>19)</sup> we extend their report in two respects. We discovered IDO1 inhibitors by a more efficient HTVS method. Comparing to NRB04258, the compound **9** had a scaffold with naphthalene group that may give rise to more potent for the compound **9** with  $IC_{50}$  value of  $8.8\ \mu M$ . The proposed virtual screening cascade takes advantage of computational efficiency of a fuzzy pharmacophore model and accuracy of molecular docking, and it may be adapted elsewhere.

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**Conflict of Interest** The authors declare no conflict of interest.

**Supplementary Materials** The online version of this article contains supplementary materials.

### References

- Hanahan D., Weinberg R. A., *Cell*, **144**, 646–674 (2011).
- Zamanakou M., Germeis A. E., Karanikas V., *Immunol. Lett.*, **111**, 69–75 (2007).
- Uyttenhove C., Pilotte L., Theate I., Stroobant V., Colau D., Parmentier N., Boon T., Van den Eynde B. J., *Nat. Med.*, **9**, 1269–1274 (2003).
- Yamamoto S., Hayaishi O., *J. Biol. Chem.*, **242**, 5260–5266 (1967).
- Munn D. H., Mellor A. L., *J. Clin. Invest.*, **117**, 1147–1154 (2007).
- Novitskiy S. V., Moses H. L., *Cancer Discov.*, **2**, 673–675 (2012).
- Ting K. K., Brew B. J., Guillemin G. J., *J. Neuroinflamm.*, **6**, 36 (2009).
- Cady S. G., Sono M., *Arch. Biochem. Biophys.*, **291**, 326–333 (1991).
- Yu C. J., Zheng M. F., Kuang C. X., Huang W. D., Yang Q., *J. Alzheimer's Dis.*, **22**, 257–266 (2010).
- Yang S., Li X., Hu F., Li Y., Yang Y., Yan J., Kuang C., Yang Q., *J. Med. Chem.*, **56**, 8321–8331 (2013).
- Combs A. P., Yue E. W., Sparks R. B., Zhu W., 1,2,5-Oxadiazoles as inhibitors of indoleamine 2,3-dioxygenase, U.S. Patent 8822511 (2012).
- Dolušić E., Frederick R., *Expert Opinion on Therapeutic Patents*, **23**, 1367–1381 (2013).
- John S., Thangapandian S., Sakkiah S., Lee K. W., *Eur. J. Med. Chem.*, **45**, 4004–4012 (2010).
- Dolušić E., Larrieu P., Blanc S., Sapunarić F., Pouyez J., Moineaux L., Colette D., Stroobant V., Pilotte L., Colau D., Ferain T., Fraser G., Galleni M., Frère J. M., Masereel B., Van den Eynde B., Wouters J., Frédérick R., *Eur. J. Med. Chem.*, **46**, 3058–3065 (2011).
- Peng Y. H., Ueng S. H., Tseng C. T., Hung M. S., Song J. S., Wu J. S., Liao F. Y., Fan Y. S., Wu M. H., Hsiao W. C., Hsueh C. C., Lin S. Y., Cheng C. Y., Tu C. H., Lee L. C., Cheng M. F., Shia K. S., Shih C., Wu S. Y., *J. Med. Chem.*, **59**, 282–293 (2016).
- Tojo S., Kohno T., Tanaka T., Kamioka S., Ota Y., Ishii T., Kamimoto K., Asano S., Isobe Y., *ACS Med. Chem. Lett.*, **5**, 1119–1123 (2014).
- Waring M. J., Arrowsmith J., Leach A. R., Leeson P. D., Mandrell S., Owen R. M., Pairaudeau G., Pennie W. D., Pickett S. D., Wang J., Wallace O., Weir A., *Nat. Rev. Drug Discov.*, **14**, 475–486 (2015).
- Davis B. J., Erlanson D. A., *Bioorg. Med. Chem. Lett.*, **23**, 2844–2852 (2013).
- Röhrig U. F., Majjigapu S. R., Chambon M., Bron S., Pilotte L., Colau D., Van den Eynde B. J., Turcatti G., Vogel P., Zoete V., Michielin O., *Eur. J. Med. Chem.*, **84**, 284–301 (2014).