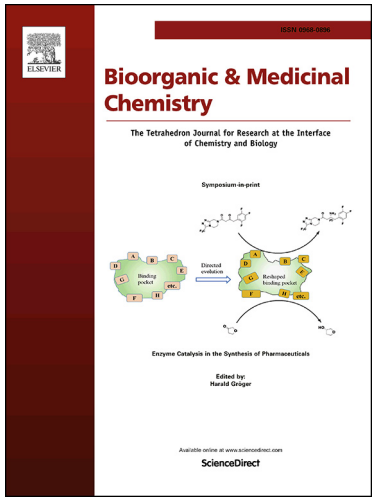

4, 6-Substituted-1*H*-Indazoles as Potent IDO1/TDO Dual Inhibitors

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4, 6-Substituted-1*H*-Indazoles as Potent IDO1/TDO Dual Inhibitors

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ABSTRACT

Indoleamine 2, 3-dioxygenase 1 (IDO1) and tryptophan 2, 3-dioxygenase (TDO) are constitutively overexpressed in many types of cancer cells and exert important immunosuppressive functions. In this article, a series of 4, 6-substituted-1*H*-indazole derivatives were synthesized and evaluated the inhibitory activities against IDO1 and TDO, as well as their structure-activity relationships (SARs). Among these, compound **35** displayed the most IDO1 inhibitory potency with an IC_{50} value of 0.74 μM in an enzymatic assay and 1.37 μM in HeLa cells. Quantitative analysis of the Western blot results indicated that **35** significantly decreased the INFγ-induced IDO1 expression in a concentration-dependent manner. In addition, **35** showed promising TDO inhibition with an IC_{50} value of 2.93 μM in the enzymatic assay and 7.54 μM in A172 cells. Moreover, compound **35** exhibited *in vivo* antitumor activity in the CT26 xenograft model. These findings suggest that 1*H*-indazole derivative **35** is a potent IDO1/TDO dual inhibitor, and has the potential to be developed for IDO1/TDO-related cancer treatment.

Keywords: Indoleamine 2,3-dioxygenase 1 (IDO1); Tryptophan 2,3-dioxygenase (TDO); 1*H*-Indazoles; Dual inhibitor; Cancer immunotherapy; Antitumor activity.

1. Introduction

The interactions between the immune system and the developments of tumors are dynamic and complex [1, 2]. Recently, immunotherapeutic approaches in the field of cancer research are rapidly expanding worldwide.. CTLA4 and PD-1/PD-L1 inhibitors are proven to be effective immunotherapeutic targets, and their inhibitors are currently available to breach immune suppression established by tumor cells and treat a variety of cancers, such as non-small-cell lung cancer, metastatic melanoma, and more[3-5]. However, the response rates of these agents as the single treatment are less than 40%. It is necessary to develop a novel therapeutic approach to increase these response rates and improve treatment results in cancer patients.

Tryptophan catabolism is closely related to antitumor immune suppression in several types of human cancers. Over 90% of *L*-tryptophan utilized by humans is processed via the kynurenine pathway [6, 7]. Indoleamine 2,3-dioxygenase 1 (IDO1) and tryptophan 2,3-dioxygenase (TDO) are heme-containing enzymes that are expressed in tumor cells and are responsible for the first and rate-limiting step of the kynurenine pathway in the tumor tissue. This process depletes tryptophan and produces metabolites, such as kynurenine, kynurenic acid, and quinolinic acid [8]. The two enzymes are constitutively overexpressed in tumor cells and tumor environmental cells, and exert important immunosuppressive functions such as suppressing effector T cells and promoting regulatory T cells (Tregs) [9-11]. Furthermore, overexpression of IDO1 and TDO is associated with reduced survival rates in cancer patients [12]. These findings indicate that IDO1 and TDO are promising targets in the field of tumor immunotherapy [13].

Although IDO1 and TDO catalyze the same biochemical reaction, they have different tissue distributions, catalytic properties and physiological roles, while the sequence identity between TDO and IDO1 is only 10% [14]. IDO1 can be expressed constitutively or can be strongly induced by IFN-γ in many types of cancer cells [15]. However, certain IDO-negative tumor types can also catabolize tryptophan. TDO is a structurally distinct enzyme, which is considered to regulate tryptophan levels in several types of human cancer, such as malignant gliomas and hepatocellular carcinoma [8, 10, 16]. These findings suggested that both enzymes were involved in the immunosuppressive activity of tryptophan catabolism in many types of cancers.

The development of IDO1 inhibitors are continuing in both academic research and pharmaceutical companies. Several new IDO1 inhibitors have been discovered via structure-based design, high throughput screening (HTS), and natural product screening [17]. Five small-molecule compounds (Indoximod, Epacadostat, Navoximod, BMS-986205 and PF-06840003) have entered clinical trials as single agents, as well as in combination with chemotherapeutics agents and immunological checkpoints mediators (Figure 1) [18-21]. Epacadostat (INBC024360) could increase response rates and improve results regarding PD-1 inhibitors in phase I/II trials [22, 23]. However, in the phase III trial involving melanoma, INBC024360in combination with Pembrolizumab did not exhibit a satisfactory inhibitory effect [24]. This results have hindered other phase trials developments of IDO1 inhibitors. However, the potential of IDO1 inhibitors as anticancer agents to improve cancer immunosuppression is undeniable.

Contrary to IDO1 inhibitors, no TDO inhibitors have been subject to clinical development, and only a few studies exist in this regard (Figure 1). More than 70 indole derivatives were synthesized based on the structure of 680C91[25], which is a TDO inhibitor with limited efficacy and oral bioavailability. Furthermore, LM10 displayed TDO inhibition (Ki: 5.5 μM), high

selectivity and good oral bioavailability [26]. Naphthotriazolediones were identified as novel TDO inhibitors by using structure-based virtual screening, that comprised of homology modeling and ligand−support binding site optimization [27]. Using HTS, aminoisoxazoles were identified as potent TDO inhibitors and structure−activity relationships (SARs) analysis revealed that both the amino group and the isoxazole moiety are fundamental in TDO inhibitory activity [28]. Yang *et al.* developed optimal enzymatic and cellular assays of TDO activity, and suggested that tryptanthrins have the potential to be developed as promising molecules for TDO-related target therapy [29].

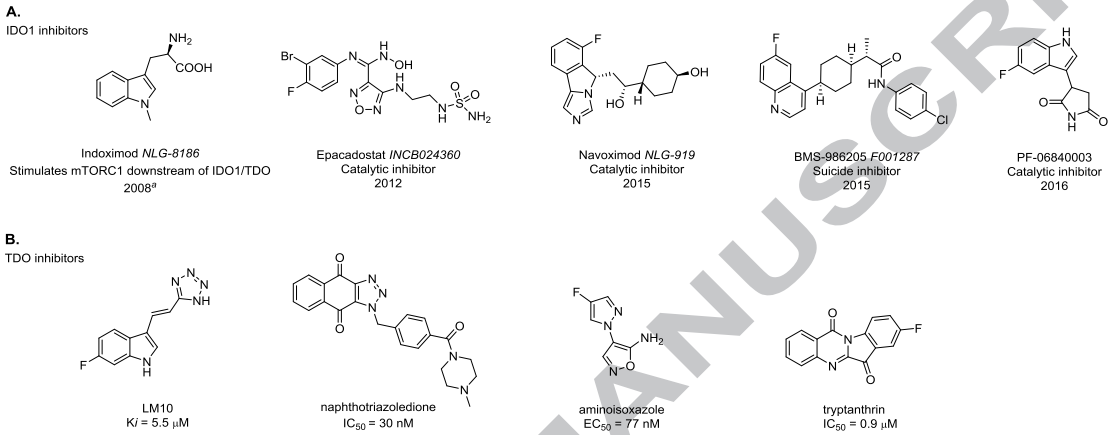


Figure 1. (A) Five IDO1 inhibitors in the clinical phase; (B) TDO inhibitors reportedly in development.

a. The year of clinical entry.

As repored previously, 4,6-substituted-1*H*-indazoles are a novel class of IDO1 inhibitors, and one representative compound is shown in Figure 2 [30]. LWQ-84, which exhibited potent IDO inhibitory activity with an IC_{50} value of 5.3 μM in the enzymatic assay, was selected for further evaluation. However, LWQ-84 displayed poor IDO1 inhibition in the cell-based assay and almost no anticancer effect in the CT26 xenograft model, which could be mainly due to the moderate potency against IDO1. Moreover, LWQ-84 did not inhibit TDO in any way. To identify more potent IDO1 and TDO inhibitors, a series of 4, 6-substituted-1*H*-indazole derivatives were designed and synthesized and their inhibitory activities against IDO1 and TDO were investigated. The SARs were also be discussed. Moreover, thehe most active compound was evaluated *in vivo* to determine its anticancer effect.

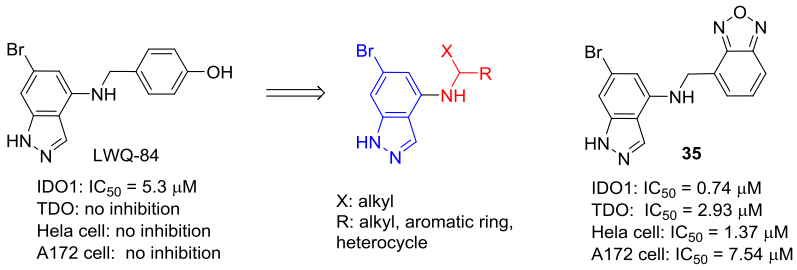
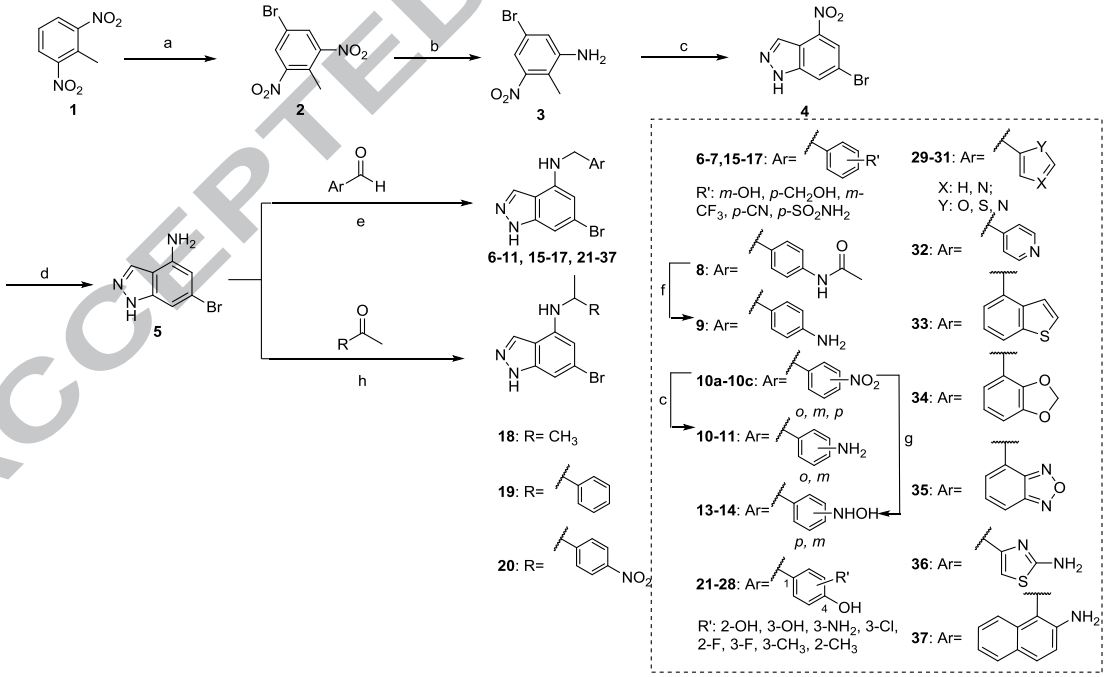


Figure 2. The design strategy for 1*H*-indazole derivatives.

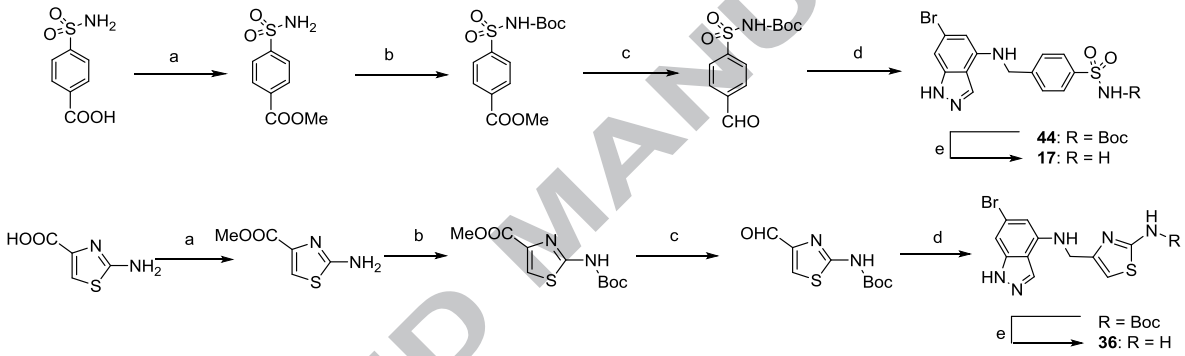
2. Chemistry

A series of 6-bromo-1*H*-indazole-4-amines with various substituent groups at C4 position were synthesized to study the SARs of IDO1 and TDO via the synthetic routes outlined in Schemes 1-6. A general strategy for the synthesis of compounds **6-37** is shown in Scheme 1. 5-Bromo-2-methyl-1, 3-dinitrobenzene (**2**) was synthesized by the halogenation of 2-methyl-1,3-dinitrobenzene (**1**) with DBDMH. A selective reductive reaction of **2** with 3 equiv. iron powder and hydrochloric acid was subsequently performed in MeOH/dioxane to produce aniline **3** in satisfying yield. Then, 6-bromo-4-nitro-1*H*-indazole **4** was synthesized from aniline **3** with sodium nitrite and acetic acid according to the reported method [31], which involved diazotization and cyclization in one pot. The ^{1}H NMR of **4** exhibited the characteristic three peaks corresponding to H3, H7, and H5, respectively of the indazole scaffold. A reductive reaction of **4** with iron powder was performed for the production of the key 6-bromo-1*H*-indazole-4-amine (**5**). The 1*H*-indazole-4-amines (**6-11**, **13-17**, and **21-37**) were synthesized via the condensation of amine **5,** and substituted the aryl aldehydes using diethyl 2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate (Hantzsch ester, DHP) as the hydrogen source and TFA as the acidic catalyst in dry DCM [32, 33]. Among these compounds, amine **9** was prepared via acidic hydrolysis from acetamide **8** in quantitative yields; amines **10-11** were prepared via a reductive reaction with iron powder from the corresponding nitro-compounds **10a-10b**; hydroxyamines **13–14** were prepared via a reductive reaction with zinc powder from the corresponding nitro-compounds **10b-10c**. The 1*H*-indazole-4-amines (**18-20**) were synthesized by the condensation of amine **5** and acetones via a microwave reaction using 4Å MS as the dehydrator in dry dichloroethane.

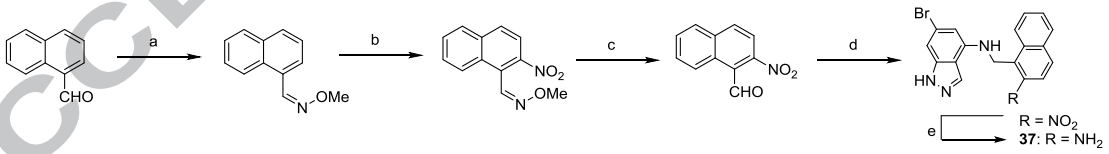


Scheme 1. Reagents and conditions: (a) DBDMH, conc.H_2SO_4, rt, 8h; (b) Fe, HCl, MeOH/dioxane (3:1), 80°C, 4 h; (c) $NaNO_2$, AcOH, H_2O, 0°C, 45 min; (d) Fe, NH_4Cl, EtOH/H_2O (3:1), 80°C, 1 h; (e) DHP, TFA, DCM/MeOH (3:1), 45°C, 4 h; (f) $SOCl_2$, MeOH, 65°C, 2 h; (g) Zn, NH_4Cl, dioxane/H_2O (1:1), rt.,12 h; (h) DCE, 4Å MS, MW., 9 h; trichlorosilane, DMF, DCM, 0°C, 1 h.

The optimized synthesis of the compounds **17**, **36-37** are shown in Scheme 2-3, since the aryl aldehyde starting material of these compounds could not be obtained from commercial sources. Methyl esters were produced by esterifying 2, 4-Sulfamoylbenzoic acid and 2-aminothiazole-4-carboxylic acid , respectively. The Boc group subsequently protected the amino groups of esters , which were then reduced with DIBAL-H to produce aldehydes, that interacted with amine **5**. Finally, they were hydrolyzed in TFA to afford the title compounds **17** and **36** (Scheme 2). The aldehyde group of 1-naphthaldehyde was protected with methoxylamine. Oxime was subsequently nitrified in the presence of $Pd(OAc)_2$, $AgNO_2$ and $K_2S_2O_8$ to produce the nitro-compound, which was hydrolyzed in *p*-TsOH and formalin to afford 2-nitro-1-naphthaldehyde. The reductive amination and reductive reaction were performed in sequence to produce the title compound **37** (Scheme 3).



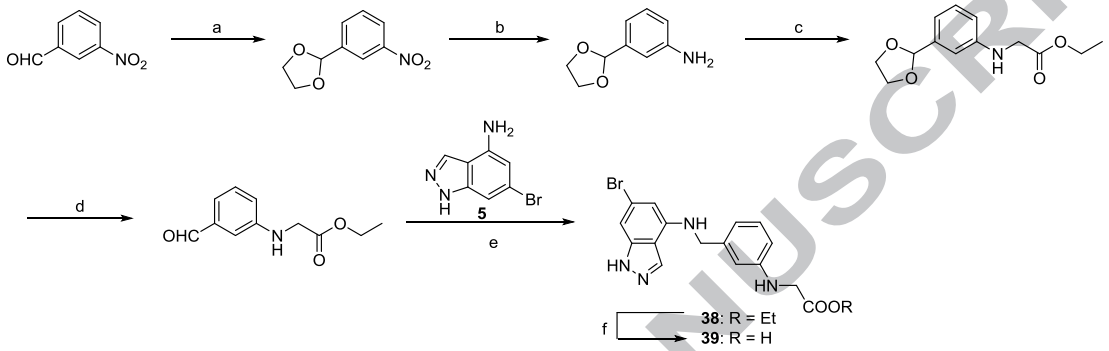
Scheme 2. Reagents and conditions: (a) $SOCl_2$, MeOH, 40°C, 2 h; (b) $(Boc)_2O$, DMAP, Et_3N, DCM, rt., 1.5 h; (c) DIBAL-H, DCM, -78°C, 2 h; (d) **5**, DHP,TFA, DCM/MeOH (3:1), 45°C, 4 h; (e) TFA, DCM, rt., 1 h.



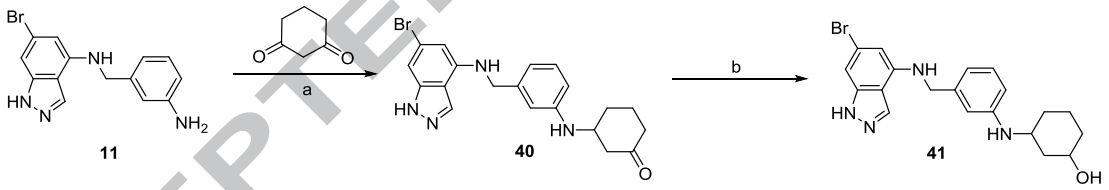
Scheme 3. Reagents and conditions: (a) methoxylamine hydrochloride, NaOH, 90% EtOH, 80°C, 4 h; (b) $Pd(OAc)_2$, $AgNO_2$, $K_2S_2O_8$, DCE, 110°C, 48 h; (c) *p*-TsOH, 7% formalin, THF, 100°C, 4 h; (d) DHP,TFA, DCM/MeOH (3:1), 45°C, 4 h; (e) Fe, NH_4Cl, $EtOH/H_2O$ (3:1), 80°C, 1 h.

The synthesis of the compounds **38-43**, which contain long aryl side-chains substituted in the C4 position of the indazole are outlined in Schemes 4-6. The aldehyde group of 3-nitrobenzaldehyde was protected with ethylene glycol in the presence of *p*-TsOH. Then, the reductive reaction, nucleophilic substitution and hydrolysis reaction were performed in sequence to produce ethyl (3-formylphenyl)glycinate in high yield. Ester **38** was obtained by the condensation reaction between an aldehyde and an amine **5** and was then hydrolyzed to afford acid **39** (Scheme 4). Alcohol **41** was obtained from amine **11** via Hantzsch ester-involved reductive amination with

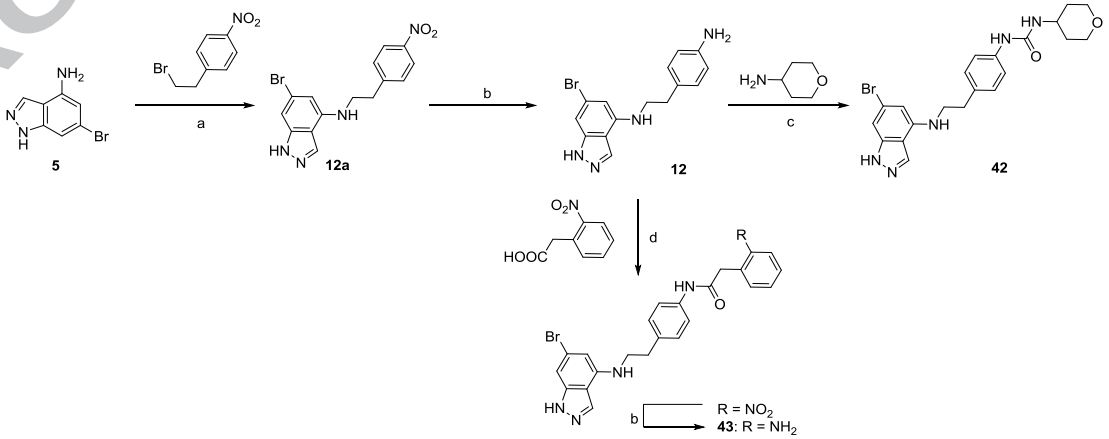
cyclohexane-1,3-dione and the $NaBH_4$-involved reductive reaction (Scheme 5). Amine **12** was prepared from amine **5** using a two-step procedure involving base-mediated nucleophilic substitution and a Fe-involved reductive reaction. Urea **42** was prepared via the condensation reaction between amine **5** and tetrahydro-2*H*-pyran-4-amine in the presence of trichloromethyl carbonate, while amine **43** was prepared via EDCI-mediated amide formation and the Fe-involved reductive reaction (Scheme 6).



Scheme 4. Reagents and conditions: (a) ethylene glycol, *p*-TsOH, toluene, 110°C, 12 h; (b) Fe, NH_4Cl, EtOH/H_2O (3:1), 80°C, 1 h; (c) ethyl bromoacetate, K_2CO_3, DMF, rt.,8 h; (d) 3N HCl, THF, rt., 6 h; (e) DHP,TFA, DCM/MeOH (3:1), 45°C, 4 h; (f) NaOH , H_2O/EtOH (1:1), 100°C, 30 min.



Scheme 5. Reagents and conditions: (a) DHP,TFA, DCM/MeOH (3:1), 45°C, 4 h; (b) $NaBH_4$, EtOH, rt., 12 h.



Scheme 6. Reagents and conditions: (a) $CsCO_3$, DMF, 65°C, 4 h; (b) Fe, NH_4Cl, EtOH/H_2O (3:1),

80°C, 1 h; (c) Trichloromethyl carbonate, DIEA, DCM, rt., 3 h; (d) HATU, DIEA, EDCI, DCM, rt., 4 h.

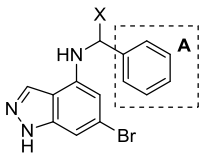
3. Results and discussion

3.1. The SARs Studies

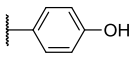
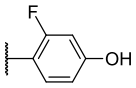
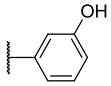
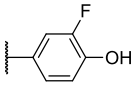
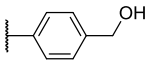
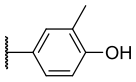
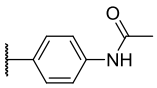
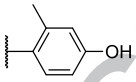
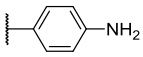
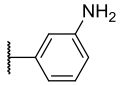
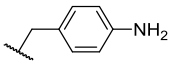
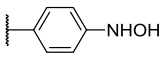
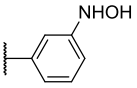
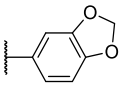
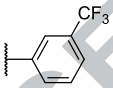
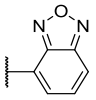
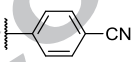
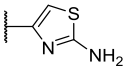
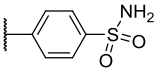
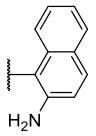
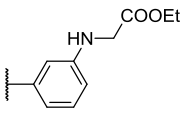
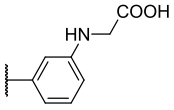
As reported previously, the effective interactions of the polar group in the 4-position of 1*H*-indazole derivatives with key residues of hydrophobic Pocket B ensured the IDO1 inhibitory activities [30]. In this study, various polar groups were employed to replace the hydroxyl group of LWQ-84to synthesiz compounds **6**-**17** (Table 1) using the routes shown in Scheme 1-2. A comparison of the inhibitory activities of the compounds LWQ-84, **6,** and **7** indicated that the hydroxyl group at the para-position of the phenyl A moiety increased the potency of IDO1 while decreasing that of TDO. The compounds **8**-**14**, which had respective levels of amino, formamide, and hydroxylamino in the phenyl A moiety, displayed weak potency against both IDO1 and TDO. The compounds **15** and **17**, which with respective levels of trifluoromethyl, aminosulfonyl, exhibited improved potency against TDO than IDO1, while cyano-compound **16** showed remarkable IDO1/TDO dual inhibitory potency. The N-phenylethyl-indazol-4-amines (**19** and **20**) were less effective than the corresponding benzyl-indazol-4-amines [30], respectively. Therefore, this result revealed that the different linkers in the 4-position of the indazoles mianly affected the IDO1 inhibitory activity. The influence of various disubstituents at the 2, 4- and 3, 4-position of the phenyl A moiety on the inhibitory activity were also investigated based on the structure of LWQ-84, and compounds **21**-**28** were synthesized using the routes shown in Scheme 1. However, only compound **25** exhibited IDO1 inhibitory activity equivalent to that of LWQ-84. This finding suggested that the IDO1 inhibitory activity declined in conjunction with an increase in the number of substituents in the phenyl A moiety.

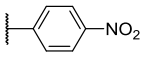
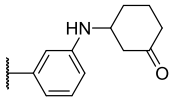
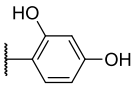
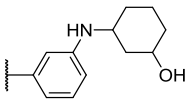
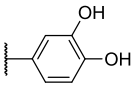
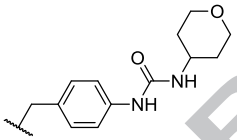
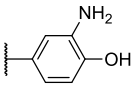
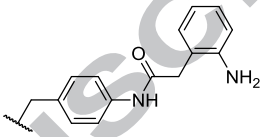
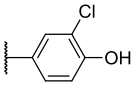
Various heteroaromatic rings were then used to replace the phenyl A of LWQ-84 and compounds **29**-**37** were synthesized using the routes shown in Scheme 1-3. Among these compounds, **35** with benzo-oxadiazole in the 4-position of the indazole, showed the most inhibitory potency against both IDO and TDO, with IC_{50} values of 0.74 μM and 2.94 μM, respectively (Figure 3). Compound **36**, which had 2-amino-thiazole in the 4-position of indazole, exhibited selective potency against TDO. Given the interspace and residue of pocket B of IDO1 and TDO, compounds **38**-**43** with extended aromatic rings were synthesized using the routes shown in Scheme 4-6, to explore additional interactions with the protein. However, the activities of these compounds were unsatisfactory.

Table 1. Inhibitory rate (at 10μM) against IDO1 and TDO.



ID	X	A	IDO Inh%[c]	TDO Inh%	ID	X	A	IDO Inh%	TDO Inh%

LWQ-84 30	H		70±2[a]	NI[b]	**25**	H		69±1	NI
6	H		45±1	55±2	**26**	H		52±3	26±1
7	H		45±2	56±3	**27**	H		28±2	30±4
8	H		48±5	NI	**28**	H		60±5	19±1
9	H		3±1	24	**29**	H		29±2	64±1
10	H		36±2	NI	**30**	H		54±2	53±5
11	H		31±1	NI	**31**	H		16±2	54±2
12	H		2±1	NI	**32**	H		54±4	55±1
13	H		NI	6±2	**33**	H		3±3	56±3
14	H		53±2	NI	**34**	H		68±6	62±4
15	H		16±2	56±1	**35**	H		91±2	85±3
16	H		70±3	73±2	**36**	H		NI	73±2
17	H		20±2	75±2	**37**	H		15±2	NI
18	CH_3	CH_3	47±2	NI	**38**	H		29±2	NI
19	CH_3		14±6	NI	**39**	H		57±1	NI

20	CH_3		17±1	56±5	**40**	H		52±6	46±2
21	H		40±2	NI	**41**	H		52±2	50±2
22	H		50±1	NI	**42**	H		NI	20±1
23	H		32±2	NI	**43**	H		3±6	29±2
24	H		60±5	19±2					

a. Each compound was tested in triplicate; the data are presented as the mean ± SD.

b. NI: no inhibition.

c. The IC_{50} value of Epacadostat, measured as 67 nM in this study, was consistent with the previous value of 72nM..

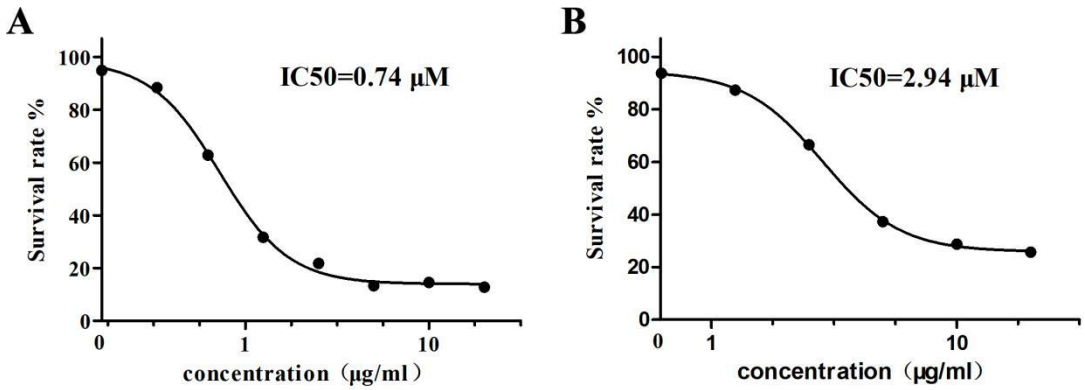


Figure 3. IC_{50} curves of **35** with IDO1 and TDO (A) **35** with IDO1 (B) **35** with TDO

*3.2. The effect of **35** on IDO1 Activity and Expression in HeLa cells.*

HeLa cells were treated with different concentrations of LWQ-84 and **35** for 2 h to study the cellular IDO1 inhibitory activity. The results indicated that the compound **35** inhibits IDO1 activity in a concentration-dependent manner with an IC_{50} value of 1.37 μM (Figure 4A). For comparison, LWQ-84 were tested but no apparent inhibition was evident in HeLa cells.

The effect of compound 35 on IDO1 expression was also investigated. The findings indicated

that theINFγ treatment of HeLa cells increased the IDO1 expression while the pre-treatment of these cells with **35** significantly decreased the INFγ-induced IDO1 expression in a concentration-dependent manner (Figure 5A), while a reduction in the expression of housekeeping gene (GAPDH) were also observed. However, quantitative analysis of the Western blot results indicated that **35** exhibited the inhibitory effect on IDO1 expression. LWQ-84 exhibited almost no effect on IDO1 expression (Figure 5B).

*3.3. The effect of **35** on TDO Activity in A172 cells.*

The cellular TDO inhibitory activity was evaluated using TDO high-expression A172 cells. Following an 8 h treatment, the amount of Kyn in the cell culture medium was measured. The results showed that compound **35** inhibited Kyn production in a concentration-dependent manner with an IC_{50} value of 7.54 μM (Figure 4B). This data indicated that compound **35** could inhibit TDO activity in a concentration-dependent manner and exhibited better selectivity for IDO1 in cellular assays compared to TDO.

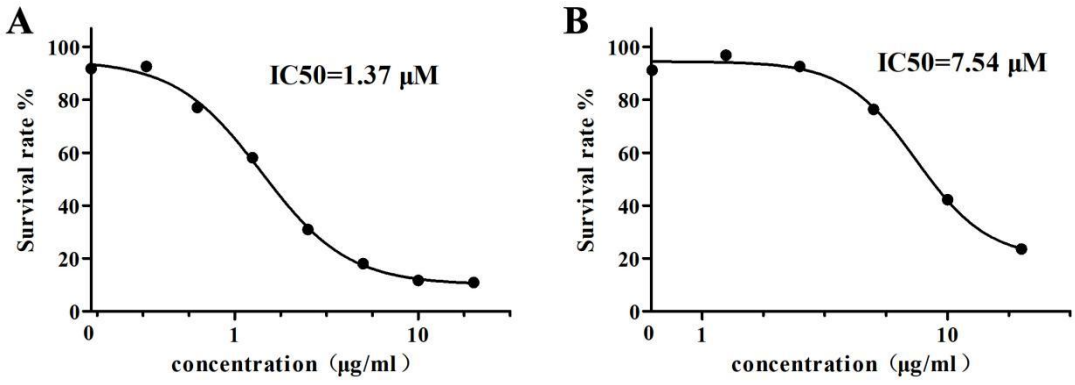


Figure 4. Compound **35** inhibits IDO1 and TDO activity in tumor cells. (A) **35** inhibits IDO1 activity in INFγ-induced HeLa cells in a concentration-dependent manner; (B) **35** inhibits TDO activity in A172 cells in a concentration-dependent manner.

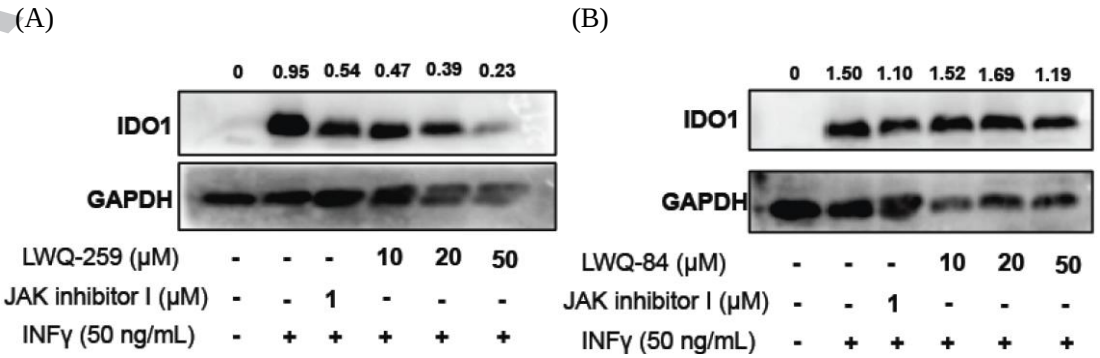
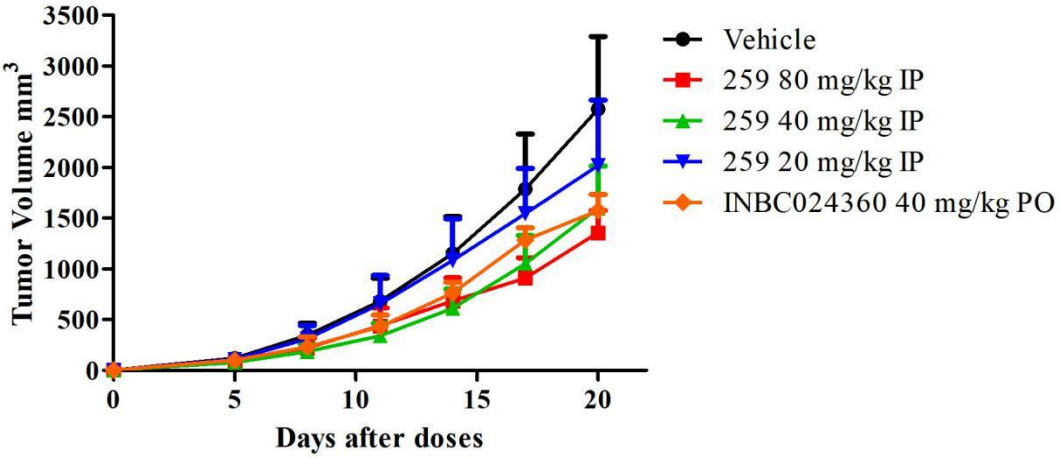


Figure 5. The effects of **35** and LWQ-84 on IDO1 protein expression evaluated by Western blot: (A) quantitative analysis of Western blot results indicated that **35** significantly decreased the

INFγ-induced IDO1 expression in a concentration-dependent manner: (B) LWQ-84 exhibited almost no effect on IDO1 expression.

*3.4. The effect of **35** on the Tumor Growth in CT26 Tumor-Bearing Mice.*

To study the *in vivo* antitumor activity of compound **35**, CT26 xenograft-bearing Balb/c mice were subcutaneously (s.c.) injected with doses of 80 mg/kg, 40 mg/kg, and 20 mg/kg, respectively. INBC024360 at a dose of 20 mg/kg was applied as a positive control. As shown in Figure 6, compound **35** significantly inhibited tumor growth compared with the vehicle. After treatment for 18 d, the compound **35** exhibited significant tumor suppressor characteristics with an anti-tumor rate of 47.3% at a dose of 80 mg/kg. Compound **35** also moderately inhibited the CT26 tumor growth with anti-tumor rates of 38.1 % and 21.7% at doses of 40 mg/kg and 20 mg/kg, respectively. At a dose of 20 mg/kg, INBC024360 inhibited tumor growth at an anti-tumor rate of 39.2 %. Therefore, compound **35** exhibited *in vivo* antitumor activity in the CT26 xenograft model.



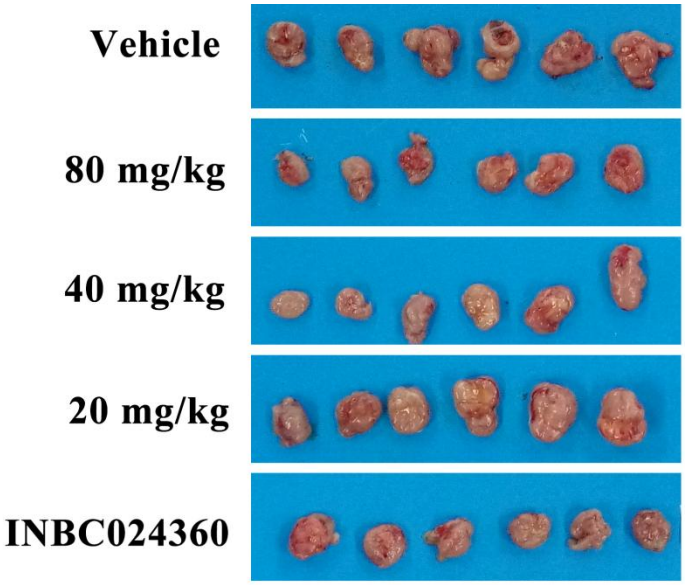


Figure 6. The *in vivo* effects of compound **35** in the CT26 tumor xenografts. CT26 cells were s.c. injected into the flank of Balb/C mice with 1×10^6 cells/ mouse. A daily s.c. administration of compound **35** and INBC024360 was initiated after 4 d of incubation.

4. Conclusion

In conclusion, 38 4, 6-substituted-1*H*-indazole derivatives were successfully synthesized and tested to evaluate their inhibitory efficacy against human IDO1 and TDO. Compound **35** displayed the most IDO1 inhibitory potency against both IDO and TDO, with IC_{50} values of 0.74 μM and 2.94 μM, respectively;**35** exhibited the IDO1 inhibitory activity in the HeLa cell assay with an IC_{50} value of 1.37 μM and TDO inhibitory activity in the A172 cell assay with an IC_{50} value of 7.54 μM. **35** exhibited the selectivity for IDO1 compared to TDO in the cellular assay. In addition, the pre-treatment of HeLa cells with **35** significantly decreased the INFγ-induced IDO1 expression in a concentration-dependent manner. Quantitative analysis of Western blot results indicated **35** exhibited an inhibitory effect on IDO1 expression. Finally, compound **35** exhibited *in vivo* antitumor activity in the CT26 xenograft model. Due to the complexity and multi-pathway nature of immunological tolerance, lower immunological tolerance and higher anticancer efficacy might be achieved by a potent IDO1/TDO dual inhibitor via two distinct tryptophan catabolism mediated events.

5. Experimental section

5.1. Chemistry methods

All chemicals were commercially available unless otherwise specified. Reactions were performed using oven-dried glassware under an atmosphere of nitrogen. The removal of solvent

was carried with a rotary evaporator and vacuum pump. All progress was monitored by thin layer chromatography with silica gel precoated glass and fluorescent indicator. Flash column chromatography was carried with silica gel and solvent system. ^{1}H NMR and ^{13}C-NMR spectra were measured on a Bruker Avance 400 and Bruker DPX 300 spectrometer, respectively. Melting points were measured on an electrothermal melting point apparatus. High-resolution mass spectrometry was measured by the micrOTOF-Q II 10203 mass spectrometer with AP-ESI ion source.

5.1.1. 6- Bromo-4-nitro-1*H*-indazole (4)

Synthesized from 2-methyl-1, 3-dinitrobenzene **1** according to our reported method [34] in 60% for three steps; yellow solid, 99.2% HPLC purity; mp: 238-239°C; The NMR data is in agreement with the reported value.

5.1.4. General procedure 1: Hantzschester-involved reductive amination

TFA (0.1 equiv.) were added to the solution of substituted anilins (1.0 equiv.), different aromaticaldehydes (1.2 equiv.), and Hantzsch ester (1.2 equiv.) in DCM/MeOH (3:1) at room temperature, and the reaction was warmed to 45°C and reacted for about 4 h. After completion (monitored by TLC), the solution was adjusted to pH 7-8 by addition of $NaHCO_3$, and the crude residue was obtained by concentrating in vacuo. Finally, the crude residue was purified by column chromatography to give the intermediate or target compounds in high yield.

5.1.5. General procedure 2: Fe-mediated reduction reaction

A slurry of nitro-compound (1.0equiv.), iron powder (5.0 equiv.) and ammonium chloride (0.5 equiv.) in EtOH/H_2O (3:1) was heated at 80°C for 1 h. The mixture was filtered through celite. The filtrate was extracted with EA. The combined organic layers were washed with brine, dried over anhydrous Na_2SO_4. The solvent was evaporated and the crude product was charged on a silica gel column chromatography to afford amine in high yield.

5.1.6. 6-Bromo-1*H*-indazol-4-amine (5).

Using 6-bromo-4-nitro-1*H*-indazole (**4**), the compound **5** was obtained as a brown solid by the general procedure 2 in 95% yield, 99.1% HPLC purity; mp: 179-181°C; The NMR data is in agreement with the reported value [30].

5.1.7. 3-(((6-Bromo-1*H*-indazol-4-yl)amino)methyl)phenol (6).

Using 3-hydroxybenzaldehyde, synthesized from **5** by the general procedure 1 in 80% yield, 99.3% HPLC purity. mp: 182-183°C; ^{1}H-NMR (DMSO-d_6) δ 12.97 (br, 1H), 9.35 (br, 1H), 8.23 (s, 1H), 7.13 (t, J = 7.7 Hz, 1H), 6.88-6.72 (m, 3H), 6.68-6.59 (m, 1H), 6.03 (s, 1H), 5.77 (s, 1H), 4.36 (d, J = 9.4 Hz, 2H).^{13}C-NMR (DMSO-d_6) δ 158.0, 143.2, 142.2, 141.4, 132.6, 129.9, 121.8, 118.0, 114.2, 114.0, 112.6, 101.1, 100.2, 46.3. HRMS: 318.0233$(M+H)^+$ (Calcd.for $C_{14}H_{12}BrN_3O$: 318.0237).

5.1.8. (4-(((6-Bromo-1*H*-indazol-4-yl)amino)methyl)phenyl)methanol (7).

Using 4-(hydroxymethyl)benzaldehyde, synthesized from **5** by the general procedure 1 in 82% yield, 98.5% HPLC purity. mp: 188-189°C; ^{1}H-NMR (d_6-DMSO) δ 12.84 (s, 1H), 8.22 (s, 1H),

7.34-7.27 (m, 5H), 6.82 (s, 1H), 6.03 (s, 1H), 5.15 (s, 1H), 4.47 (s, 2H), 4.42 (d, *J* = 3.1 Hz, 2H).[13]C-NMR (d_6-DMSO) δ 143.2, 142.2, 141.6, 138.2, 132.6, 127.2, 127.1, 121.7, 112.6, 101.2, 100.3, 63.2, 46.2. HRMS: 332.0392 $(M+H)^+$ (Calcd.for $C_{15}H_{14}BrN_3O$: 332.0393).

5.1.9. *N*-(4-(((6-Bromo-1*H*-indazol-4-yl)amino)methyl)phenyl)acetamide (8).

Using N-(4-formylphenyl)acetamide, synthesized from **5** by the general procedure 1 in 73% yield, 98.7% HPLC purity. mp: 205-206°C; [1]H-NMR (DMSO-d_6) δ 12.84 (s, 1H), 9.93 (s, 1H), 8.22 (s, 1H), 7.54 (d, *J* = 8.5 Hz, 2H), 7.30 (d, *J* = 8.5 Hz, 2H), 7.25 (t, *J* = 5.9 Hz, 1H), 6.82 (s, 1H), 6.05 (s, 1H), 4.37 (d, *J* = 5.8 Hz, 2H), 2.03 (s, 3H). HRMS: 359.0499 $(M+H)^+$ (Calcd.for $C_{16}H_{15}BrN_4O$: 359.0502).

5.1.10. *N*-(4-Aminobenzyl)-6-bromo-1*H*-indazol-4-amine (9).

Synthesized from **8** according to our reported method [35] in 46% yield; yellow solid; 97.8% HPLC purity. mp: 178-179°C; [1]H-NMR (DMSO-d_6) δ 12.87 (s, 1H, indazole-NH), 8.19 (s, 1H), 6.89-6.77 (m, 3H), 6.47-6.39 (m, 3H), 4.11 (s, 2H), 4.00 (s, 2H). HRMS: 317.0397$(M+H)^+$ (Calcd.for $C_{14}H_{13}BrN_4$: 317.0396) .

5.1.11. *N*-(2-Aminobenzyl)-6-bromo-1*H*-indazol-4-amine (10).

Using nitrobenzaldehyde, **10a-10c** were obtained from **5** by the general procedure 1. The compound **10** was obtained from 6-bromo-N-(2-nitrobenzyl)-1*H*-indazol-4-amine (**10a**) by the general procedure 2, 71% for two steps, 97.9% HPLC purity. mp: 165-166°C; [1]H-NMR (DMSO-d_6) δ 12.83 (s, 1H), 8.22 (s, 1H), 7.11-7.05 (m, 2H), 6.98 (t, *J* = 7.8 Hz, 1H), 6.85 (s, 1H), 6.69 (d, *J* = 7.7 Hz, 1H), 6.54 (t, *J* = 7.4 Hz, 1H), 6.08 (s, 1H), 5.03 (s, 2H), 4.26 (d, *J* = 5.6 Hz, 2H).[13]C-NMR (DMSO-d_6) δ 146.7, 143.4, 142.1, 132.7, 128.0, 128.0, 122.1, 121.8, 116.5, 115.4, 112.6, 101.3, 100.2, 43.58. HRMS: 317.0398 $(M+H)^+$ (Calcd.for $C_{14}H_{13}BrN_4$: 317.0396).

5.1.12. *N*-(3-Aminobenzyl)-6-bromo-1*H*-indazol-4-amine (11).

The ompound **11** was syntheszied using the method similar to that for **10**, 72% for two steps, 98.6% HPLC purity. mp: 172-173°C; [1]H-NMR (DMSO-d_6) δ 12.81 (s, 1H), 8.23 (s, 1H), 7.22 (t, *J* = 4.6 Hz, 1H), 6.98 (t, *J* = 7.6 Hz, 1H), 6.82 (s, 1H), 6.58 (s, 1H), 6.53 (d, *J* = 7.2 Hz, 1H), 6.44 (d, *J* = 7.7 Hz, 1H), 6.03 (s, 1H), 5.04 (s, 2H), 4.29 (d, *J* = 5.3 Hz, 2H).[13]C-NMR (DMSO-d_6) δ 149.3, 143.4, 142.1, 140.5, 132.7, 129.4, 121.8, 114.9, 113.0, 112.6, 101.1, 100.0, 46.8. HRMS: 317.0393$(M+H)^+$（Calcd.for $C_{14}H_{13}BrN_4$: 317.0396）.

5.1.13. *N*-(4-Aminophenethyl)-6-bromo-1*H*-indazol-4-amine (12).

Synthesized from **5** according to our reported method [36] in 64.5% for two steps, 98.8% HPLC purity. mp: 203-204°C; [1]H-NMR (DMSO-d_6) δ 8.08 (s, 1H), 6.89 (m, 1H), 6.86 (d, *J* = 8.3 Hz, 2H), 6.46 (d, *J* = 8.3 Hz, 2H), 6.27 (d, *J* = 1.2 Hz, 1H), 6.12 (s, 1H), 4.87 (s, 2H), 4.36 (t, *J* = 7.5 Hz, 2H), 2.90 (t, *J* = 7.5 Hz, 2H).[13]C-NMR (DMSO-d_6) δ 147.4, 143.8, 141.7, 131.9, 129.6, 125.6, 121.6, 114.4, 113.0, 104.4, 99.3, 50.4, 35.1. HRMS: 331.0547 $(M+H)^+$ (Calcd.for $C_{15}H_{15}BrN_4$: 331.0553).

5.1.14. 6-Bromo-*N*-(4-(hydroxyamino)benzyl)-1*H*-indazol-4-amine (13).

Synthesized from **10c** according to our reported method [36] in 46.7% yield; yellow solid, 98.2% HPLC purity. mp: 205-206°C; ^{1}H-NMR (DMSO-d_6) δ12.80 (s, 1H), 8.22 (s, 1H), 7.11-6.99 (m, 3H), 6.80 (s, 1H), 6.54 (d, *J* = 8.3 Hz, 2H), 6.08 (s, 1H), 4.23 (d, *J* = 5.7 Hz, 2H). ^{13}C-NMR (DMSO-d_6) δ 148.1, 143.3, 142.1, 132.8, 128.5, 126.3, 121.8, 114.3, 112.6, 101.2, 99.9, 46.4. HRMS: 333.0349(M+H)$^+$ (Calcd.for $C_{14}H_{13}BrN_4O$: 333.0346) .

5.1.15. 6-Bromo-*N*-(3-(hydroxyamino)benzyl)-1*H*-indazol-4-amine (14).

The compound **14** was obtained using the method similar to that for **13** in 48.9% yield, 96.5% HPLC purity. mp: 182-183°C; ^{1}H-NMR (DMSO-d_6) δ 12.87 (d, *J* = 6.8 Hz, 1H), 8.37-7.90 (m, 3H), 7.76-7.33 (m, 3H), 6.86 (d, *J* = 5.2 Hz, 1H), 6.10 (d, *J* = 10.7 Hz, 1H), 4.57 (dd, *J* = 26.3, 6.0 Hz, 2H). HRMS: 333.0346(M+H)$^+$ (Calcd.for $C_{14}H_{13}BrN_4O$: 333.0346).

5.1.16. 6-Bromo-*N*-(3-(trifluoromethyl)benzyl)-1*H*-indazol-4-amine (15).

Using 3-(trifluoromethyl)benzaldehyde, synthesized from **5** by the general procedure 1 in 87% yield, 98.1% HPLC purity. mp: 124-125°C; ^{1}H-NMR (DMSO-d_6) δ 12.87 (s, 1H), 8.22 (s, 1H), 7.76 (s, 1H), 7.70 (d, *J* = 7.3 Hz, 1H), 7.65 – 7.54 (m, 2H), 7.36 (t, *J* = 6.1 Hz, 1H), 6.85 (s, 1H), 6.08 (d, *J* = 1.1 Hz, 1H), 4.55 (d, *J* = 6.1 Hz, 2H). ^{13}C-NMR (DMSO-d_6) δ 142.9, 142.2 141.6, 132.6, 131.6, 129.9 129.8 129.5 126.1, 123.4, 121.8 112.7, 101.3, 100.7, 45.9. HRMS: 370.0156(M+H)$^+$ (Calcd.for $C_{15}H_{11}BrF_3N_3$: 370.0161) .

5.1.17. 4-(((6-Bromo-1*H*-indazol-4-yl)amino)methyl)benzonitrile (16).

Using 4-formylbenzonitrile, synthesized from **5** by the general procedure 1 in 85% yield, 95.3% HPLC purity. mp: 201-202°C; ^{1}H-NMR (DMSO-d_6) δ 12.89 (s, 1H), 8.23 (s, 1H), 7.82 (d, *J* = 8.3 Hz, 2H), 7.58 (d, *J* = 8.3 Hz, 2H), 7.41 (t, *J* = 6.1 Hz, 1H), 6.87 (s, 1H), 6.02 (s, 1H), 4.57 (s, 2H). HRMS: 327.0241(M+H)$^+$ (Calcd.for $C_{15}H_{11}BrN_4$: 327.0240) .

5.1.18. 4-(((6-Bromo-1*H*-indazol-4-yl)amino)methyl)benzenesulfonamide (17).

To the solution of 4-sulfamoylbenzoic acid (200 mg, 0.53 mmol) in MeOH (8 mL) added $SOCl_2$ (1348 μL, 1.84 mmol) at 0°C. The mixture was stirred at 40°C for 2 h, and then concentrated. Ester (214 mg, 1.00 mmol) and $(Boc)_2O$ (238 mg, 1.09 mmol) were dissolved in DCM (8 mL). Et_3N (138 μL, 1 mmol) and DMAP (12.2 mg, 0.1 mmol) were added and the mixture was stirred at rt. for 1.5 h. The solution was concentrated and purified to afford methyl 4-(N-(tert-butoxycarbonyl)sulfamoyl)benzoate. DIBAL-H (2 mL, 2 mmol) was added slowly to methyl benzoate (300 mg, 1.00 mmol) in DCM (8 mL) at -78°C and the mixture was stirred at -78°C for 2 h. The reaction was quenched by MeOH (2 mL), and then warmed to 0°C and added 10% citric acid under stirring. The mixture was extracted with DCM, and the organics were washed, dried, concentrated and purified to afford 4-formylbenzenesulfonamide. Using 4-formylbenzenesulfonamide, the compound **44** was obtained from **5** by the general procedure as above. To the solution of **44** (95 mg, 0.20 mmol) in DCM (4 mL) added TFA (300 μL, 0.04 mmol). The mixture was stirred at rt. for 1 h. The solution was adjusted to pH 7-8 by $NaHCO_3$. The mixture was extracted with EA, and the organics were washed, dried, concentrated and purified to afford **17**, 54% yield for five steps, 94.0% HPLC purity. mp: 210-212°C; ^{1}H-NMR (DMSO-d_6) δ 12.88 (s, 1H), 8.23 (s, 1H), 7.85 (d, *J* = 8.4 Hz, 2H), 7.62 (d, *J* = 8.4 Hz, 2H), 7.44 (t, *J* = 6.0 Hz, 1H), 6.85 (s, 1H), 5.97 (s, 1H), 4.58 (d, *J* = 6.0 Hz, 2H). HRMS:381.0015(M+H)$^+$

（Calcd.for $C_{14}H_{13}BrN4_3O_2S$: 381.0015）.

5.1.19. 6-bromo-*N*-isopropyl-1*H*-indazol-4-amine (18).

Synthesized from **5** according to our reported method [36] in 60% yield; 96.9% HPLC purity. mp: 180-181°C; ^{1}H-NMR (DMSO-d_6) δ 12.78 (s, 1H), 8.21 (s, 1H), 6.80 (s, 1H), 6.30 (d, *J* = 7.8 Hz, 1H), 6.12 (s, 1H), 3.72 (m, 1H), 1.23 (d, *J* = 6.3 Hz, 6H). ^{13}C-NMR (DMSO-d_6) δ 142.6, 142.2, 132.8, 122.0, 112.5, 100.8, 99.6, 43.7, 22.7. HRMS: 254.0289 $(M+H)^+$（Calcd.for $C_{10}H_{12}BrN_3$: 254.0287）.

5.1.20. 6-Bromo-*N*-(1-phenylethyl)-1*H*-indazol-4-amine (19).

Synthesized from **5** according to our reported method [36] in 42% for two steps, 97.5% HPLC purity. mp: 189-190°C; ^{1}H-NMR ($CDCl_3$) δ 10.34 (br, 1H), 8.04 (d, *J* = 0.9 Hz, 1H), 7.46-7.34 (m, 4H), 7.33-7.25 (m, 2H), 6.97 (t, *J* = 1.1 Hz, 1H), 6.17 (d, *J* = 1.2 Hz, 1H), 4.73-4.64(m, 1H), 1.65 (d, *J* = 6.5 Hz, 3H). HRMS: 316.0442 $(M+H)^+$ (Calcd.for $C_{15}H_{14}BrN_3$: 316.0449).

5.1.21. 6-Bromo-*N*-(1-(4-nitrophenyl)ethyl)-1*H*-indazol-4-amine (20).

Synthesized from **5** according to our reported method [36] in 30% for two steps, 96.9% HPLC purity. mp: 192-193°C; ^{1}H-NMR (DMSO-d_6) δ 12.85 (s, 1HH), 8.35 (s, 1H), 8.27-8.12 (m, 2H), 7.78-7.59 (m, 2H), 7.14 (d, *J* = 7.1 Hz, 1H), 6.82 (s, 1H), 5.89 (s, 1H), 4.96-4.80 (m, 1H), 1.55 (d, *J* = 6.8 Hz, 3H). ^{13}C-NMR (DMSO-d_6) δ 154.1, 146.9, 142.1, 141.9, 132.9, 127.6, 124.3, 121.5, 112.6, 102.2, 100.8, 52.0, 24.3. HRMS: 361.0293$(M+H)^+$ (Calcd.for $C_{15}H_{13}BrN_4O_2$: 361.0295).

5.1.22. 4-(((6-Bromo-1*H*-indazol-4-yl)amino)methyl)benzene-1,3-diol (21).

Using 2, 4-dihydroxybenzaldehyde, synthesized from **5** by the general procedure 1 in 49.5% yield, 90.0% HPLC purity. mp: 222-223°C; ^{1}H-NMR (DMSO-d_6) δ12.66 (s, 1H), 9.47 (s, 1H), 8.95 (s, 1H), 8.10 (s, 1H), 6.37 (s, 1H), 6.32 (d, *J* = 2.2 Hz, 1H), 6.19 (d, *J* = 8.3 Hz, 1H), 5.99 (dd, *J* = 8.3, 2.2 Hz, 1H), 5.91 (s, 1H), 3.97 (s, 2H). ^{13}C-NMR (DMSO-d_6) δ 156.7, 155.9, 141.6, 133.1, 128.8, 128.6, 123.7, 116.7, 113.3, 108.8, 106.2, 105.6, 102.7, 49.1. HRMS: 334.0180$(M+H)^+$ (Calcd.for $C_{14}H_{12}BrN_3O_2$: 334.0186).

5.1.23. 4-(((6-Bromo-1*H*-indazol-4-yl)amino)methyl)benzene-1,2-diol (22).

Using 3, 4-dihydroxybenzaldehyde, synthesized from **5** by the general procedure 1 in 89% yield, 97.0% HPLC purity. mp: 152-153°C; ^{1}H-NMR (DMSO-d_6) δ 12.81 (s, 1H), 8.87 (s, 1H), 8.75 (s, 1H), 8.22 (s, 1H), 7.17 (t, *J* = 5.4 Hz, 1H), 6.85-6.74 (m, 2H), 6.73-6.60 (m, 2H), 6.05 (s, 1H), 4.25 (d, *J* = 5.6 Hz, 2H).^{13}C-NMR (DMSO-d_6) δ 145.7, 144.6, 143.3, 142.1, 132.7, 130.5, 121.8, 118.4, 115.9, 114.9, 112.6, 101.2, 100.0, 46.2. HRMS: 334.0174$(M+H)^+$ (Calcd.for $C_{14}H_{12}BrN_3O_2$: 334.0186) .

5.1.24. 2-Amino-4-(((6-Bromo-1*H*-indazol-4-yl)amino)methyl)phenol (23).

Using 3-amino-4-hydroxybenzaldehyde, synthesized from **5** by the general procedure 1 in 91% yield, 96.1% HPLC purity. mp: 182-183°C; ^{1}H-NMR (DMSO-d_6) δ 12.80 (s, 1H), 8.90 (s, 1H), 8.23 (s, 1H), 7.13 (t, *J* = 5.5 Hz, 1H), 6.80 (s, 1H), 6.67-6.54 (m, 2H), 6.43 (dd, *J* = 7.9, 1.7 Hz, 1H), 6.04 (s, 1H), 4.53 (s, 1H), 4.21 (d, *J* = 5.7 Hz, 1H).^{13}C-NMR (DMSO-d_6) δ 143.4, 142.1, 137.0, 132.8, 130.4, 121.8, 115.6, 114.6, 113.5, 112.5, 101.1, 99.8, 46.6. HRMS: Calcd.for:

333.0348(M+H)$^+$ ($C_{14}H_{13}BrN_4O$: 333.0351).

5.1.25. 4-(((6-Bromo-1*H*-indazol-4-yl)amino)methyl)-2-chlorophenol (24).

Using 3-chloro-4-hydroxybenzaldehyde, synthesized from **5** by the general procedure 1 in 96.3% yield, 96.6% HPLC purity. mp: 186-187°C; ^{1}H-NMR (DMSO-d_6) δ 12.84 (s, 1H), 10.06 (s, 1H), 8.22 (s, 1H), 7.35 (d, *J* = 2.0 Hz, 1H), 7.23-7.13 (m, 2H), 6.95 (d, *J* = 8.3 Hz, 1H), 6.85 (s, 1H), 6.09 (d, *J* = 0.8 Hz, 1H), 4.33 (d, *J* = 5.9 Hz, 2H).^{13}C-NMR (DMSO-d_6) δ 152.4, 143.0, 142.1, 132.7, 131.6, 128.9, 127.4, 121.8, 120.0, 117.0, 112.6, 101.3, 100.4, 45.5. HRMS: 351.9844(M+H)$^+$ (Calcd.for $C_{14}H_{11}BrClN_3O$: 351.9847).

5.1.26. 4-(((6-Bromo-1*H*-indazol-4-yl)amino)methyl)-3-fluorophenol (25).

Using 2-fluoro-4-hydroxybenzaldehyde, synthesized from **5** by the general procedure 1 in 92.5% yield, 99% HPLC purity. mp: 182-183°C; ^{1}H-NMR (DMSO-d_6) δ 12.83 (s, 1H), 9.82 (s, 1H), 8.22 (s, 1H), 7.21 (t, *J* = 8.6 Hz, 1H), 7.08 (t, *J* = 5.6 Hz, 1H), 6.85 (s, 1H), 6.67-6.55 (m, 2H), 6.12 (d, *J* = 0.9 Hz, 1H), 4.33 (d, *J* = 5.6 Hz, 2H). HRMS: 336.0132(M+H)$^+$ (Calcd.for $C_{14}H_{11}BrFN_3O$: 336.0142).

5.1.27. 4-(((6-Bromo-1*H*-indazol-4-yl)amino)methyl)-2-fluorophenol (26).

Using 3-fluoro-4-hydroxybenzaldehyde, synthesized from **5** by the general procedure 1 in 69.1% yield, 99% HPLC purity. mp: 196-197°C; ^{1}H-NMR (DMSO-d_6) δ 12.85 (s, 1H), 9.74 (s, 1H), 8.22 (s, 1H), 7.20 (t, *J* = 5.8 Hz, 1H), 7.14 (dd, *J* = 12.2, 1.7 Hz, 1H), 7.02 (dd, *J* = 8.3, 1.3 Hz, 1H), 6.92 (t, *J* = 8.6 Hz, 1H), 6.85 (s, 1H), 6.08 (s, 1H), 4.33 (d, *J* = 5.9 Hz, 2H). HRMS: 336.0132(M+H)$^+$ (Calcd.for $C_{14}H_{11}BrFN_3O$: 336.0142) .

5.1.28. 4-(((6-Bromo-1*H*-indazol-4-yl)amino)methyl)-2-methylphenol (27).

Using 4-hydroxy-3-methylbenzaldehyde, synthesized from **5** by the general procedure 1 in 69.2% yield, 97.8% HPLC purity. mp: 192-193°C; ^{1}H-NMR (DMSO-d_6) δ 12.84 (s, 1H), 9.23 (s, 1H), 8.23 (s, 1H), 7.13 (t, *J* = 5.2 Hz, 1H), 7.09 (s, 1H), 7.01 (d, *J* = 8.0 Hz, 1H), 6.83 (s, 1H), 6.75 (d, *J* = 8.1 Hz, 1H), 6.08 (s, 1H), 4.27 (d, *J* = 5.4 Hz, 2H), 2.11 (s, 3H). HRMS: 332.0391(M+H)$^+$ (Calcd.for $C_{15}H_{14}BrN_3O$: 332.0393).

5.1.29. 4-(((6-Bromo-1*H*-indazol-4-yl)amino)methyl)-3-methylphenol (28).

Using 4-hydroxy-2-methylbenzaldehyde, synthesized from **5** by the general procedure 1 in 64.6% yield, 97.1% HPLC purity. mp: 193-194°C; ^{1}H-NMR (DMSO-d_6) δ 8.30 (s, 1H), 7.08 (d, *J* = 8.2 Hz, 1H), 6.86 (s, 1H), 6.65 (d, *J* = 2.4 Hz, 1H), 6.56 (dd, *J* = 8.2, 2.4 Hz, 1H), 6.11 (s, 1H), 4.26 (d, *J* = 7.2 Hz, 1H), 2.26 (s, 3H). HRMS: 332039(M+H)$^+$ (Calcd.for $C_{15}H_{14}BrN_3O$: 332.0393).

5.1.30. 6-Bromo-*N*-(furan-2-ylmethyl)-1*H*-indazol-4-amine (29).

Using furan-2-carbaldehyde, synthesized from **5** by the general procedure 1 in 63.7%% yield, 92.8% HPLC purity. mp: 195-196°C; ^{1}H-NMR (DMSO-d_6) δ 12.84 (s, 1H), 8.20 (s, 1H), 7.61 (dd, *J* = 1.8, 0.8 Hz, 1H), 7.14 (t, *J* = 5.8 Hz, 1H), 6.87 (s, 1H), 6.43-6.40 (m, 1H), 6.36 (dd, *J* = 3.1, 0.5 Hz, 1H), 6.25 (s, 1H), 4.42 (d, *J* = 5.8 Hz, 2H). HRMS: 292.0075(M+H)$^+$ (Calcd.for $C_{12}H_{10}BrN_3O$: 292.0080).

5.1.31. 6-Bromo-*N*-(thiophen-2-ylmethyl)-1*H*-indazol-4-amine (30).

Using thiophene-2-carbaldehyde, synthesized from **5** by the general procedure1 in 69.4% yield, 97.6% HPLC purity. mp: 183-184°C; ^{1}H-NMR (DMSO-d_6) δ 12.85 (s, 1H), 8.21 (s, 1H), 7.39 (dd, *J* = 5.1, 1.1 Hz, 1H), 7.31 (t, *J* = 5.9 Hz, 1H), 7.10 (d, *J* = 2.6 Hz, 1H), 7.03-6.97 (m, 1H), 6.88 (s, 1H), 6.23 (s, 1H), 4.63 (d, *J* = 5.9 Hz, 2H). HRMS: 307.9850(M+H)$^+$ (Calcd.for $C_{12}H_{10}BrN_3S$: 307.9852).

5.1.32. *N*-((1H-Imidazol-5-yl)methyl)-6-bromo-1*H*-indazol-4-amine (31).

Using 1*H*-imidazole-5-carbaldehyde, synthesized from **5** by the general procedure 1 in 66.7% yield , 98.1% HPLC purity. mp: 194-195°C; ^{1}H-NMR (DMSO-d_6) δ 12.82 (s, 1H), 11.95 (s, 1H), 8.22 (s, 1H), 7.62 (d, *J* = 0.6 Hz, 1H), 6.99 (s, 1H), 6.94 (t, *J* = 5.4 Hz, 1H), 6.84 (s, 1H), 6.24 (s, 1H), 4.31 (d, *J* = 5.4 Hz, 2H). HRMS: 292.0189(M+H)$^+$ (Calcd.for $C_{11}H_{10}BrN_5$:292.0198).

5.1.33. 6-Bromo-*N*-(pyridin-4-ylmethyl)-1*H*-indazol-4-amine (32).

Using isonicotinaldehyde, synthesized from **5** by the general procedure 1 in 63.2% yield, 97.3% HPLC purity. mp: 135-136°C; ^{1}H-NMR (DMSO-d_6) δ 12.89 (s, 1H), 8.52 (d, *J* = 5.9 Hz, 2H), 8.23 (s, 1H), 7.42-7.34 (m, 3H), 6.87 (s, 1H), 6.01 (s, 1H), 4.51 (d, *J* = 6.1 Hz, 2H). HRMS: 303.0238(M+H)$^+$ (Calcd.for $C_{13}H_{11}BrN_4$: 303.0240).

5.1.34. *N*-(Benzo[*b*]thiophen-4-ylmethyl)-6-bromo-1*H*-indazol-4-amine (33).

Using benzo[*b*]thiophene-4-carbaldehyde, synthesized from **5** by the general procedure 1 in 72% yield, 90.5% HPLC purity. mp: 204-205°C; ^{1}H-NMR (DMSO-d_6) δ 12.70 (s, 1H), 7.90 (d, *J* = 7.8 Hz, 2H), 7.84-7.75 (m, 1H), 7.54-7.39 (m, 2H), 7.39-7.11 (m, 4H), 6.64 (d, *J* = 46.7 Hz, 1H), 4.76 (d, *J* = 4.9 Hz, 2H). HRMS: 358.0000 (M+H)$^+$ (Calcd.for $C_{16}H_{12}BrN_3S$: 358.0008).

5.1.35. *N*-(Benzo[d][1,3]dioxol-5-ylmethyl)-6-bromo-1*H*-indazol-4-amine (34).

Using benzo[*d*][1,3]dioxole-4-carbaldehyde, synthesized from **5** by the general procedure 1 in 82% yield, 96.5% HPLC purity. mp: 208-209°C; ^{1}H-NMR (DMSO-d_6) δ 12.80 (s, 1H), 8.23 (s, 1H), 7.21 (t, *J* = 5.8 Hz, 1H), 6.96 (s, 1H), 6.88 (d, *J* = 0.8 Hz, 2H), 6.85 (s, 1H), 6.09 (s, 1H), 5.99 (s, 2H), 4.35 (d, *J* = 6.0 Hz, 2H). ^{13}C-NMR (DMSO-d_6) δ 147.8, 146.7, 143.1, 142.1 , 133.7 , 132.7 , 121.8 , 120.6 , 112.6 , 108.6 , 108.0 , 101.3, 100.3 , 46.2. HRMS: 332.0032(M+H)$^+$ (Calcd.for $C_{15}H_{12}BrN_3O_2$: 332.0035).

5.1.36. *N*-(Benzo[c][1,2,5]oxadiazol-4-ylmethyl)-6-bromo-1*H*-indazol-4-amine (35).

Using benzo[*c*][1,2,5]oxadiazole-4-carbaldehyde, synthesized from **5** by the general procedure 1 in 92% yield, 97.5% HPLC purity. mp: 195-196°C; ^{1}H-NMR (DMSO-d_6) δ 12.90 (s, 1H), 8.25 (s, 1H), 7.95 (d, *J* = 8.7 Hz, 1H), 7.59 (dd, *J* = 9.0, 6.6 Hz, 1H), 7.52-7.36 (m, 2H), 6.89 (s, 1H), 6.14 (d, *J* = 0.8 Hz, 1H), 4.85 (d, *J* = 5.8 Hz, 2H). ^{13}C-NMR (DMSO-d_6) δ 149.7, 148.7, 142.8, 142.1, 133.3, 132.6, 128.9 , 128.6 , 121.7, 114.9, 112.6, 101.2, 100.9, 43.0. HRMS: 344.0137(M+H)$^+$ (Calcd.for $C_{14}H_{10}BrN_5O$: 344.0142).

5.1.37. 4-(((6-Bromo-1*H*-indazol-4-yl)amino)methyl)thiazol-2-amine (36).

Using 2-aminothiazole-4-carboxylic acid, synthesized from **5** according to the general procedure

of **17**, 60% yield for five steps. 95% HPLC purity. mp: 165-166°C; ^{1}H-NMR (DMSO-d_6) δ 12.80 (s, 1H), 8.20 (s, 1H), 7.04 (t, *J* = 5.8 Hz, 1H), 6.90 (s, 2H), 6.82 (s, 1H), 6.31 (s, 1H), 6.13 (d, *J* = 0.9 Hz, 1H), 4.20 (d, *J* = 5.7 Hz, 2H). ^{13}C-NMR (DMSO-d_6) δ 169.1, 150.1, 143.2, 142.1, 132.7, 121.8, 112.6, 102.2, 101.1, 100.1, 43.7. HRMS: 323.9916(M+H)$^+$ (Calcd.for $C_{11}H_{10}N_5S$: 323.9919).

5.1.38. *N*-((2-Aminonaphthalen-1-yl)methyl)-6-bromo-1*H*-indazol-4-amine (37).

Synthesized from **5** according to our reported method [36] in 20.6% for five steps, 97.6% HPLC purity. mp: 171-172°C; ^{1}H-NMR (DMSO-d_6) δ 12.93 (s, 1H), 8.28 (s, 1H), 7.74 (dd, *J* = 8.0, 1.5 Hz, 1H), 7.43-7.31 (m, 2H5), 7.28-7.17 (m, 3H), 7.00 (s, 1H), 6.82 (dd, *J* = 5.6, 3.2 Hz, 1H), 6.37 (s, 1H), 5.52 (s, 2H), 4.86 (d, *J* = 4.7 Hz, 2H).^{13}C-NMR (DMSO-d_6) δ 146.5, 142.5, 142.1, 136.7, 134.1, 133.0, 129.3, 128.1, 126.8, 125.4, 124.3, 121.8, 118.4, 112.9, 112.4, 102.3, 101.7, 48.7. HRMS: 367.0553 (M+H)$^+$ (Calcd.for $C_{18}H_{16}BrN_4$: 367.0541).

5.1.39. Ethyl (3-(((6-bromo-1*H*-indazol-4-yl)amino)methyl)phenyl)glycinate (38).

Synthesized from **5** according to our reported method [36] in 67% for five steps, 97.5% HPLC purity. mp: 181-182°C; ^{1}H-NMR (DMSO-d_6) δ 12.82 (s, 1H), 8.23 (s, 1H), 7.23 (t, *J* = 5.8 Hz, 1H), 7.05 (t, *J* = 7.7 Hz, 1H), 6.82 (s, 1H), 6.61 (d, *J* = 9.0 Hz, 2H), 6.41 (d, *J* = 8.0 Hz, 1H), 6.10-5.98 (m, 2H), 4.32 (d, *J* = 5.8 Hz, 2H), 4.06 (q, *J* = 7.1 Hz, 2H), 3.85 (d, *J* = 6.4 Hz, 2H), 1.15 (t, *J* = 7.1 Hz, 3H).^{13}C-NMR (DMSO-d_6) δ 171.7, 148.7, 143.4, 142.1, 140.6, 132.7, 129.4, 121.8, 115.6, 112.6, 111.2, 110.9, 101.1, 100.1, 60.7, 46.8, 45.2, 14.6. HRMS: 403.0765(M+H)$^+$ (Calcd.for $C_{18}H_{19}BrN_4O_2$: 403.0770).

5.1.40. (3-(((6-Bromo-1*H*-indazol-4-yl)amino)methyl)phenyl)glycine (39).

Synthesized from **38** according to our reported method [36] in 58% yield; yellow solid; 92.5% HPLC purity. mp: 202-203°C; ^{1}H-NMR (DMSO-d_6) δ 12.74 (br, 2H), 8.24 (s, 1H), 7.21 (t, *J* = 5.6 Hz, 1H), 7.05 (t, *J* = 7.7 Hz, 1H), 6.82 (s, 1H), 6.69-6.54 (m, 2H), 6.42 (dd, *J* = 8.0, 1.5 Hz, 1H), 6.05 (s, 1H), 4.31 (d, *J* = 5.2 Hz, 2H), 3.78 (s, 2H).^{13}C-NMR (DMSO-d_6) δ 173.1, 148.8, 143.4, 142.2, 140.5, 132.6, 129.4, 121.8, 115.5, 112.6, 111.5, 110.8, 101.1, 100.1, 46.9, 45.1. HRMS: 375.0450(M+H)$^+$ (Calcd.for $C_{16}H_{15}BrN_4O_2$: 375.0451).

5.1.41. 3-((3-(((6-Bromo-1*H*-indazol-4-yl)amino)methyl)phenyl)amino)cyclohexan-1-one (40).

Using cyclohexane-1,3-dione,synthesized as a white solid from **11** by general procedure 1 in 77.3% yield. 96.9% HPLC purity. mp: 205-206°C; ^{1}H-NMR (DMSO-d_6) δ 12.86 (s, 1H), 8.84 (s, 1H), 8.23 (s, 1H), 7.36-7.07 (m, 5H), 6.84 (s, 1H), 6.04 (s, 1H), 5.35 (s, 1H), 4.46 (d, *J* = 5.9 Hz, 2H), 2.48 (t, *J* = 6.0 Hz, 2H), 2.15 (t, *J* = 6.4 Hz, 2H), 1.96-1.78 (m, 2H), 1.00-0.96 (m, 1H). ^{13}C-NMR (DMSO-d_6) δ 196.3, 162.4, 143.1, 141.4, 139.8, 129.7, 123.5, 121.9, 121.8, 121.6, 112.6, 101.2, 98.6, 46.3, 46.2, 36.9, 29.0, 22.0. HRMS: 413.0979(M+H)$^+$ (Calcd.for $C_{20}H_{21}BrN_4O$:413.0977).

5.1.42. 3-((3-(((6-Bromo-1*H*-indazol-4-yl)amino)methyl)phenyl)amino)cyclohexan-1-ol (41).

Synthesized from **40** according to our reported method [36] in 85.2% yield; 96.7% HPLC purity. mp: 211-212°C; ^{1}H-NMR (DMSO-d_6) δ 12.86 (s, 1H), 8.84 (s, 1H), 8.22 (s, 1H), 7.36-7.08 (m, 5H), 6.83 (s, 1H), 6.03 (d, *J* = 1.2 Hz, 1H), 5.77 (s, 1H), 5.34 (s, 1H), 4.45 (d, *J* = 5.9 Hz, 2H), 2.48 (t, *J* = 6.1 Hz, 2H), 2.15 (t, *J* = 6.4 Hz, 2H), 1.92-1.81 (m, 2H), 1.00 (t, *J* = 7.1 Hz,

2H).^{13}C-NMR (DMSO-d_6) δ 196.2, 162.3, 143.1, 141.4, 139.8, 129.7, 123.5, 121.9, 121.7, 121.6, 112.6, 101.2, 98.7, 55.4, 46.3, 46.2, 36.9, 29.0, 22.0. HRMS: 415.1141(M+H)$^+$ (Calcd.for $C_{20}H_{21}BrN4O$: 415.1128).

5.1.43.1-(4-(2-((6-Bromo-1*H*-indazol-4-yl)amino)ethyl)phenyl)-3-(tetrahydro-2*H*-pyran-4-yl) urea (42).

Synthesized from **12** according to our reported method [36] in 51.4% yield; white solid; 98.8% HPLC purity; mp: 215-216°C; ^{1}H-NMR (DMSO-d_6) δ 8.25 (s, 1H), 8.07 (s, 1H), 7.25 (d, *J* = 8.5 Hz, 2H), 7.05 (d, *J* = 8.4 Hz, 2H), 6.90 (s, 1H), 6.26 (d, *J* = 1.3 Hz, 1H), 6.20-5.95 (m, 2H), 4.42 (t, *J* = 7.3 Hz, 2H), 3.92-3.54 (m, 5H), 3.49-3.27 (m, 4H), 2.99 (t, *J* = 7.3 Hz, 2H).^{13}C-NMR (DMSO-d_6) δ 154.9, 143.8, 141.8, 139.2, 132.0, 131.3, 129.5, 121.6, 118.0, 113.0, 104.5, 99.2, 66.3, 50.0, 45.7, 35.1, 33.7. HRMS: 458.1188(M+H)$^+$ (Calcd.for $C_{21}H_{24}BrN_5O_2$: 458.1192).

5.1.44. 2-(2-Aminophenyl)-*N*-(4-(2-((6-bromo-1*H*-indazol-4-yl)amino)ethyl)phenyl)acetamide (43).

Synthesized from **12** according to our reported method [36] in 52.3% yield for two steps, 98.5% HPLC purity. mp: 176-177°C; ^{1}H-NMR ($CDCl_3$) δ 7.91 (s, 1H), 7.86 (s, 1H), 7.32 (d, *J* = 8.4 Hz, 2H), 7.18-7.08 (m, 2H), 6.99 (d, *J* = 8.4 Hz, 2H), 6.82-6.71 (m, 3H), 6.41 (d, *J* = 1.2 Hz, 1H), 4.41 (t, *J* = 7.4 Hz, 2H), 4.27 (s, 2H), 3.61 (s, 2H), 3.49 (s, 1H), 3.10 (t, *J* = 7.4 Hz, 2H). HRMS: 464.1082(M+H)$^+$ (Calcd.for $C_{23}H_{22}BrN_5O$: 464.1080).

5.2. Biological assays

5.2.1. Enzymatic Assay

Human N-terminus IDO1 and TDO was expressed in *Escherichia coli.* and purified using Ni^+ affinity chromatography as described [37, 38]. The assay was performed by UV absorption using recombinant hIDO1, hTDO and *L*-tryptophan as substrate. To detect the inhibitory activity of the compounds on TDO and IDO1 enzyme, recombinant hTDO (100 nM) and hIDO (100 nM) was incubated with certain concentrations of compounds at room temperature in incubation system containing 400 mM tryptophan, 40 mM ascorbic acid, 200 μg/ml catalase, 20 μM methylene blue, Ca^{2+}, Mg^{2+}-free potassium phosphate buffered. INBC024360was used as the positive control. After 1 h incubation, 30% trichloroacetic acid was added to each system and incubated at 65°C for 15 min to stop the enzyme reaction and convert the N-formyl kynurenine to kynurenine. Then, 100 μL supernatant from each system was mixed with equal volume of acetic acid containing DMAB (dimethylaminobenzaldehyde, 3‰, w/v) and optical density was detected at 480 nm wavelength using Multiscan spectrum Mk3 (Thermo Fisher). The kynurenine concentration was determined from L-kynurenine standard curve. Finally, the data was processed with the GraphPad Prism 5.0 software. The IC_{50} value was determined by the concentration causing a half-maximal percent activity.

5.2.2. Cellular IDO1 Inhibition Assay

HeLa cells were seeded in a 24-well plate for 24 h, then added INFγ (50 ng/mL), and treated with the inhibitors for 24 h. DMSO (0.5%) and INBC024360 (25 nM) were used as negative and

positive controls respectively. The IDO1 activities were determined by measuring the concentration of *L*-kynurenine in cell culture medium. In fact, 400 μL of culture medium was mixed with 180 μL of 30% trichloroacetic acid, and centrifuged at 13.000 rpm for 10 min. The supernatant (100 μL) was transfered into a new 96 well plate, and an equal volume of freshly prepared 2% w/v p-dimethylaminobenzaldehyde in acetic acid was added. The optical density was measured at 480 nm using Multiscan spectrum Mk3 (Thermo Fisher). The kynurenine concentration was determined from L-kynurenine standard curve. Each assay was performed in triplicate and data are presented as mean ± standard deviation.

5.2.3. Cellular TDO Inhibition Assay

TDO high expressed cell line A172 was used to test the hTDO activity in a cellular context. After being seeded in 96-well plates (2 × 104 cells/well) for overnight, A172 cells were treated with certain concentrations of compounds and *L*-Trp (20 μg/ml) for 24 h. Then, cell culture medium (300 μL/well) was transferred to a tube and mixed with 90 μL TCA (30%, w/v). Next, transfer the tube into 65°C water bath for 30 min to convert the N-formyl kynurenine to kynurenine. After centrifuged at 13.000 rpm for 10 min, the supernatant (100 μL) was transferred to a new 96-well microplate and an equal volume of freshly prepared 2% w/v *p*-dimethylaminobenzaldehyde in acetic acid was added. The optical density was measured at 480 nm using Multiscan spectrum Mk3 (Thermo Fisher). The kynurenine concentration was determined from *L*-kynurenine standard curve. Finally, the data was processed with the GraphPad Prism 5.0 software.

5.2.4. Western blot analysis

HeLa cells were seeded in a 96-well plate and treated with the IDO1 inhibitors for 2 h before addition of INFγ (50 ng/mL) for another 24 h. HeLa cells were seeded in a 6-well plate, and treated with the IDO1 inhibitors (10, 20 and 50 μM) and JAK inhibitor (1 μM) for 2 hours followed by addition of 50 ng/mL of INFγ for 24 hours. DMSO, 0.5% was used as negative control. The treated cells were lysed in RIPA lysis buffer, and the same amount of protein samples were loaded on a 10% Sodium Dodecyl Sulfate-Polyacrylamide gel to conduct the electrophoresis process. Thereafter, the proteins were transferred to nitrocellulose membranes, and then blocked with 5% bovine serum albumin in Tris-buffered saline with Tween 20 (TBST). The membranes were incubated overnight at 4°C with anti-IDO1 primary antibody (Rabbit polyclonal antibody, 1:1000, #13268-1-AP, Proteintech) and GAPDH (Rabbit polyclonal antibody, 1:4000, #10494-A-AP, Proteintech), then followed by incubation with horseradish peroxidase (HRP) Goat anti-Rabbit antibody IgG (1:3000, #511203, Zen BioScience) for 90 min at room temperature. Bands were visualized by using the enhanced chemiluminescence reagent (EasySee western blot kit, TransGen Biotech) on Image Quant LAS 500 (GE Healthcare Bio Sciences AB, USA).

5.2.5. In vivo anti-tumor studies

The 7-8 week old female Balb/c mice were obtained and housed in sterile barrier facilities and fed a standard diet ad libitum. The caring of animal was consistent to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. To establish the CT26 xenograft model, each Balb/c mouse were subcutaneous injected with 100 μL 1 × 107 cells/mL CT26 single-cell suspensions. After four days incubation, the mice were randomly divided into 5 groups (6 mice each group). Three groups were daily orally dosed with ISL at the dose of 20, 40, and 80

mg/kg/day respectively. Orally intaking 5% DMSO, 20% PEG400 and 75% deionized water was used as vehicle group. INBC024360 (40 mg/kg) treated group was applied as positive control. Tumors growth were measured every 3 days with a Vernier caliper during the treatment, and the tumor volume was calculated using the following formula: volume (mm^3) = $a \times b^2/2$ (a: longest diameter (length); b: shortest diameter (width)).

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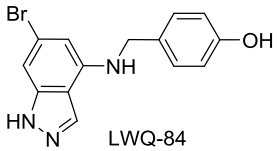
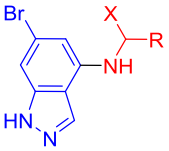
1. 38 Indazole derivatives were synthesized.
2. Compound **35** displayed the most IDO1 inhibitory potency.
3. Compound **35** was identified as an IDO1/TDO dual inhibitor.
4. Compound **35** decreased the INFγ-induced IDO1 expression.
5. Compound **35** exhibited *in vivo* antitumor activity in CT26 model.

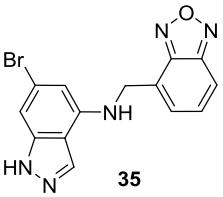


LWQ-84

IDO1: IC_{50} = 5.3 μM
TDO: no inhibition
Hela cell: no inhibition
A172 cell: no inhibition



X: alkyl
R: alkyl, aromatic ring, heterocycle



35

IDO1: IC_{50} = 0.74 μM
TDO: IC_{50} = 2.93 μM
Hela cell: IC_{50} = 1.37 μM
A172 cell: IC_{50} = 7.54 μM