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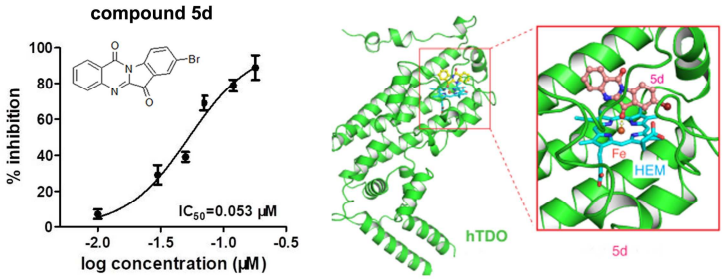

Tryptophan 2,3-Dioxygenase Inhibitory Activities of Tryptanthrin Derivatives

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Abstract

Tryptophan 2,3-dioxygenase (TDO) is becoming a promising therapeutic target due to its involvement in cancer and neurodegenerative diseases. Development of efficient TDO inhibitors is a prime strategy in disease treatment. However, the lack of a TDO inhibitor bioassay system

slows the progress of TDO inhibitor research. Herein, an active recombinant human TDO (hTDO) was prepared under optimal expression conditions, an enzymatic assay was optimized, and two cellular assays of TDO activity were developed. Then, the potential TDO inhibitory activities of nine tryptanthrin derivatives (**5a-5i**) were evaluated, and the inhibitory constants (Ki), enzymatic and cellular half maximal inhibitory concentrations (IC_{50}) were measured, and the type of inhibition was determined. The tryptanthrins had various levels of TDO inhibitory activities; tryptanthrins with a substituent at 8-position had stronger inhibitory activities than the other derivatives. Moreover, most of the compounds, except **5g** and **5h**, exhibited better inhibitory activities than the previously reported TDO inhibitor LM10. Furthermore, the molecular docking study of compounds **5c** and **5d** revealed that the O atom of the tryptanthrin ring is directed toward the heme iron (Fe) of hTDO via strong coordination interactions. These findings suggest that tryptanthrin and its derivatives have the potential to be developed as promising molecules for TDO-related target therapy.

Keywords

Tryptophan 2,3-dioxygenase, Tryptanthrin derivatives, TDO activity assay, TDO inhibitor

1. Introduction

Tryptophan (Trp) is an essential amino acid that is used by vertebrates in the synthesis of proteins and bioactive metabolites. A minor amount of Trp is catabolized to melatonin along the serotonin pathway, while over 95% of Trp is metabolized to nicotinamide adenine dinucleotide (NAD) via the kynurenine pathway [1, 2]. The kynurenine pathway generates a series of downstream metabolites such as neurotropic kynurenine (Kyn) [3] and neurotoxic quinolinic acid (QUIN) [4]; this pathway is involved in the pathological progression of cancer [5] and neurodegenerative diseases [6]. The first rate-limiting step of kynurenine pathway is catalyzed by three heme-containing enzymes including tryptophan 2,3-dioxygenase (TDO), indoleamine 2,3-dioxygenase 1 (IDO1) and indoleamine 2,3-dioxygenase 2 (IDO 2). TDO was first discovered in 1936, earlier than the discovery of IDO1 in 1967 and IDO2 in 2007 [7-10]. Although TDO, IDO1 and IDO2 catalyze the same biochemical reaction, they have different tissue distributions, catalytic properties and physiological roles. Moreover, an alignment of their sequence indicates that the sequence identity between TDO and IDO1 is only 10%, while IDO1 and IDO2 share 43%

sequence identity [11].

TDO is a homotetrameric enzyme, primarily expressed in the brain and liver [12, 13], TDO specifically catalyzes the conversion of L-Trp and its specific analogues [14, 15]. IDO1 is a monomeric enzyme expressed in various tissues, organs and certain cell types; it catalyzes the conversion of a wide range of substrates including L-Trp and 5-hydroxytryptamine [16]. IDO2 is closely related to IDO1, and is present mainly in the liver, kidney tubules, testis, and thyroid as well as in various cell lines such as pancreatic, gastric, colon and kidney carcinomas [17]. Although IDO2 exhibits much lower catalytic affinity toward L-Trp than TDO and IDO1 do, it plays a nonredundant role in the Trp metabolism [18]. IDO1 has emerged as a new significant therapeutic target due to its indispensable immunomodulatory roles in pregnancy [19, 20, 21], cancer [22], allergy [23] and central nervous system disorders such as Alzheimer's disease (AD) [24]. Many IDO1 inhibitors have been designed and several of them have entered into clinical trials including Indoximod, INCB024360, PF-06840003, GDC-0919 and F001287 [25].

Since TDO catalyzes the same reaction as IDO1 and may be an alternative drug target, TDO inhibition programs are currently receiving extensive attention. TDO positive cells inhibit T cell proliferation and alloantigen-induced T-cell activation suggesting that TDO might be involved in immunotolerance [26]. TDO is expressed in a large proportion of human tumors, and TDO expression by tumors prevents their rejection by immunized mice [27]. The constitutive expression of TDO in glioblastoma cells can upregulate the cellular level of Kyn, which activates the aryl hydrocarbon receptor (AhR), resulting in the enhancement of tumor cell growth, survival, mobility as well as escape from immune surveillance [28]. In addition, TDO plays an important role in maintaining the homeostasis of serotonin metabolism and can modulate anxiety-like behaviors. In a rat model of depressive behavior, inhibition of stress-induced hepatic TDO exhibits anti-depressant activities [29]. TDO also functions as a regulator of age-related protein toxicity in a *Caenorhabditis elegans* model, that is independent of the downstream metabolites of the kynurenine pathway [30]. TDO deficiency is reported to be neuroprotective in *C. elegans*, *Drosophila* and a mouse model of multiple sclerosis. Taken together, TDO is involved in the biology of aging and the pathological progression of cancer and neurodegenerative diseases including AD and Parkinson's disease (PD) [30, 31]. Thus, the discovery of effective TDO inhibitors is important in the treatment of cancer and neurological diseases.

In contrast to extensive studies of the IDO1 inhibitors, only a few studies of TDO inhibitors have been published, and no TDO inhibitors are clinically available. The TDO inhibitor 680C91 was discovered in 1995 by examining *in vitro* Trp catabolism using rat liver instead of the purified TDO protein [32]. The *in vivo* TDO inhibitory efficacy of 680C91 was evaluated after administration in rats, and the levels of Kyn and Trp in the brain were measured by high-performance liquid chromatography (HPLC) [32]. In 2014, Georgios Pantouris et al. reported the screening of nearly 2800 compounds using an enzymatic assay of human TDO (hTDO) activity and identified seven potent TDO inhibitors with inhibition constants (Ki) in the nanomolar or low micromolar range; however, they did not report the half maximal inhibitory concentrations (IC_{50}) of these TDO inhibitors [33]. They also claimed that NSC36398 was the first compound as having an *in vitro* selectivity for TDO over IDO 1; its Ki against TDO was approximately ~16 μM, while the Ki against IDO1 was over 100 μM. In the same year, Seegers et al. developed a high-throughput fluorescence-based screening assay for TDO and IDO1 inhibitors. Using this assay, they reported that compound **5I** and some published IDO1 inhibitors had significant cross-reactivity with TDO [34]. Using a structure-based virtual screening strategy in combination with the enzymatic assay described previously [35, 36], Wu et al. identified a potent TDO selective inhibitor, compound **11e**, with an IC_{50} of 30 nM in TDO enzymatic assay [37]. In published studies on the discovery of TDO inhibitors, expression of recombinant hTDO and conditions of the enzymatic assay of hTDO activity are not sufficiently detailed. The cellular assay of TDO activity has been routinely performed in the A172 cell line [34, 38, 39], which is limited and defective because A172 cells express all three of tryptophan-metabolizing enzymes, IDO1, IDO2 and TDO. Therefore, it is essential to develop optimal enzymatic and cellular assays of TDO activity. These developments will undoubtedly promote the progress of TDO inhibitor research.

Herein, we have optimized the conditions for expression of recombinant hTDO and the enzymatic assay of hTDO activity and established two new and reliable cellular assays of TDO activity. Using the assays, the TDO inhibitory activities of tryptanthrin derivatives were evaluated. Tryptanthrin is a natural component of the Chinese medicinal plants Polygonum tinctorium and Isatis tinctorial. In our previous studies, tryptanthrin and its eight derivatives (**5a**-**5i**) were evaluated for IDO1 and IDO2 inhibitory activities but their inhibitory activities on TDO were not

determined. The present work will provide a complete TDO inhibitor screening system which certainly benefits the future development of TDO inhibitors.

2. Results and Discussion

2.1. Purification of active recombinant human TDO

Full-length hTDO has been reported to have a high tendency to aggregate in solution and form inclusion bodies when expressed in *E. coli* [40]; hence, a truncated hTDO (aa 19-388) was used instead of the full-length hTDO for the crystallization experiment [41]. However, full-length hTDO has been used in the enzymatic assay of hTDO activity [42]. Herein, a recombinant plasmid pET28a-hTDO containing the full hTDO coding sequence and a C-terminal hexahistidine tag was constructed.

It is known that 5-aminolevulinic acid (ALA) and hemin are usually used to increase heme availability during the expression of the heme-containing proteins. To find optimal expression conditions, the effects of various concentrations of isopropyl β-D-1-thiogalactopyranoside (IPTG), ALA and hemin on recombinant hTDO activity were tested by measuring the catalytic efficiency of the crude enzyme. As shown in Fig. 1A, various concentrations of IPTG were tested (0.2 mM, 0.5 mM, 0.8 mM and 1 mM), and 0.2 mM IPTG was found to induce the highest production of Kyn, suggesting that 0.2 mM IPTG was the most beneficial concentration for recombinant hTDO expression. The combination of IPTG and hemin gave better result than the combination of IPTG and ALA (Fig. 1B). In addition, the combination of 0.5 mM hemin and 0.2 mM IPTG was more beneficial for recombinant hTDO expression compared with the combination of 0.01 mM hemin and 0.2 mM IPTG (Fig. 1B).

Recombinant hTDO was a major soluble protein in the supernatant of cell lysates. The supernatant was loaded on a Ni-NTA column and eluted with 20 mM and 40 mM imidazole solution to remove the hybrid protein. Then, recombinant hTDO was eluted from the resin using 250 mM imidazole solution. The sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the purified of recombinant hTDO is shown in Fig. 1C. Further purification could be achieved by passing the enzyme through a molecular sieve. The analysis of recombinant hTDO was resolved as a double band with molecular weights of 43 and 49 kDa (Fig. 1C), the same as predicted molecular weight (MW) (Fig. 1D).

The specific activity of recombinant hTDO was measured and the efficiency of the purification of recombinant hTDO from the bacterial pellet of 2 L of culture is summarized in Table 1. The recombinant hTDO was purified from the crude extract through the chromatography step using metal affinity column (Ni-NTA agarose) and the yield was 9 mg/L of Luria-Bertani (LB) broth; its specific activity was increased by approximately 2.8-fold. The recombinant hTDO was stored at -80□ in buffer containing 60% glycerol.

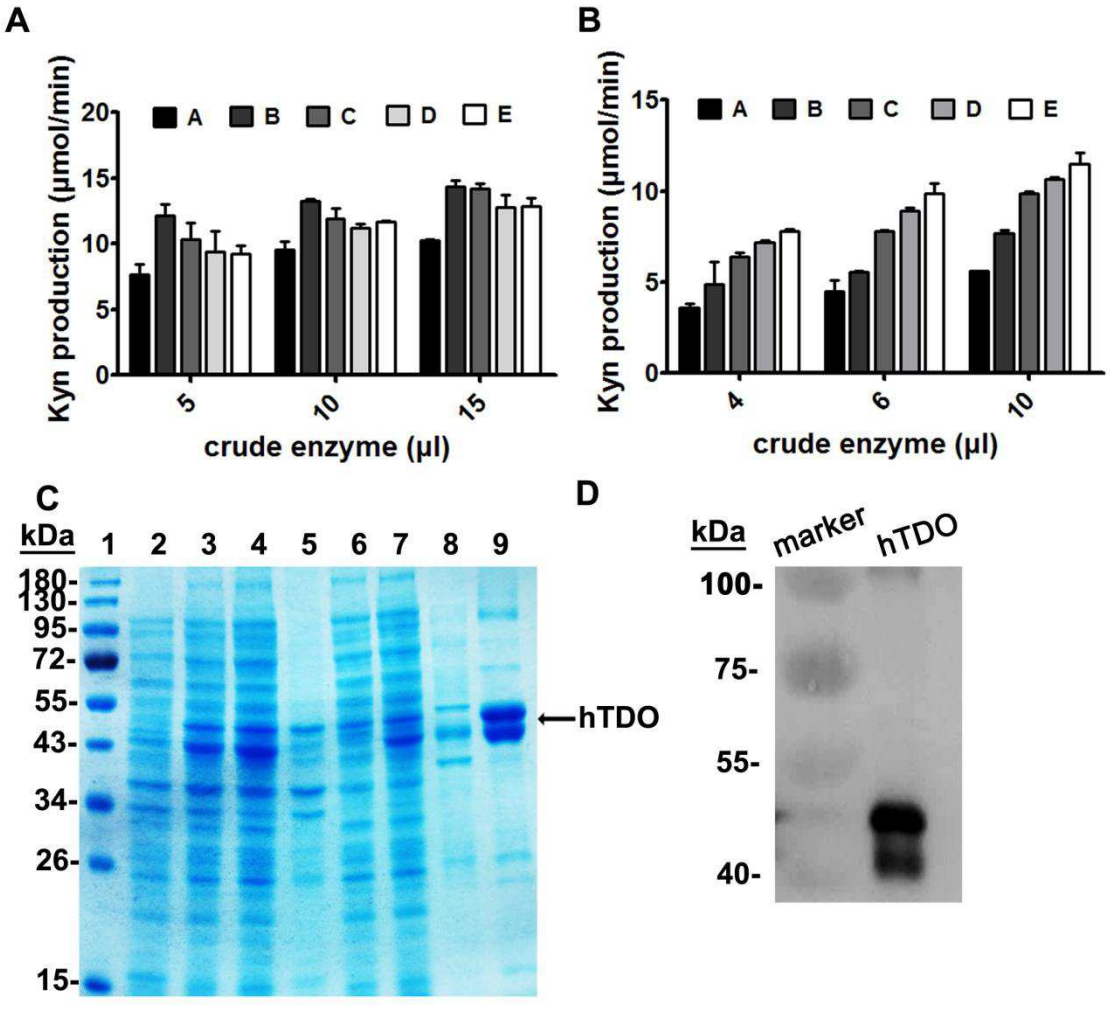


Fig. 1. Induction and purification of recombinant hTDO. A. Optimal IPTG concentration for recombinant hTDO induction. Groups A-E represent recombinant hTDO expression in *E coli* cells induced by 0.1 mM IPTG, 0.2 mM IPTG, 0.5 mM IPTG, 0.8 mM IPTG, and 1 mM IPTG, respectively. After induction, 1 L of *E. coli* cells was collected and resuspended in 30 mL of PBS; Kyn production by various volumes of cell lysates at different volume was assayed. B. Effects of ALA and hemin on recombinant hTDO expression. Groups A-E represent recombinant hTDO expression in *E coli* cells induced by 0.2 mM IPTG, 0.2 mM IPTG and 0.1 mM ALA, 0.2 mM IPTG and 0.5 mM ALA, 0.2 mM IPTG and 0.01 mM hemin, and 0.2 mM IPTG and 0.5 mM hemin, respectively. After induction,

1 L of *E. coli* cells was collected and resuspended in 30 mL of PBS, Kyn production by various volumes of cell lysates at different volume was assayed. C. SDS-PAGE analysis and Coomassie brilliant blue stained purified recombinant hTDO. Arrow indicates the predicted position of purified recombinant hTDO. Lane1: the molecular weight markers. 2: bacterially-expressed total protein without induction. 3: bacterially-expressed total protein induced by 0.2 mM IPTG and 0.5 mM hemin. 4: supernatant of the cell lysates. 5: bacterial precipitate. 6: flow-through fraction of the supernatant. 7: 20 mM imidazole eluent. 8: 40 mM imidazole eluent. 9: recombinant hTDO eluted by 250 mM imidazole buffer. D. Western blot analysis of the purified recombinant hTDO was performed using a primary polyclonal TDO2 antibody.

Table 1 Purification of recombinant human TDO.

Step	Volume (mL)	Total protein (mg)	Total activity (umol h^{-1})	Specific activity (umol $h^{-1}mg^{-1}$)	Fold
Crude extract	120	703	5382636	7656.7	1
Ni-NTA agarose	19	18	378060	21003	2.8

Purification was performed from a 2 L culture of pET28a-hTDO transformed *Escherichia coli* (*E. coli*) BL21 (DE3) and the enzyme activity was determined with L-Trp as the substrate.

2.2. Enzymatic activity of recombinant human TDO

The optimal pH conditions for high catalytic activity of recombinant hTDO have not been previously reported. Herein, we determined the optimum pH via measuring the Kyn production catalyzed by recombinant hTDO under various pH conditions. The results suggested that recombinant hTDO exhibited relatively high activity in the pH 6.5~7.5 interval and pH 7.0 was the optimal pH for recombinant hTDO activity (Supplemental Fig. S1). The enzyme kinetic parameters including the Km and Kcat values were determined by incubating recombinant hTDO with a series of concentrations of L-Trp at optimal pH 7.0 and the data are listed in Table 2. In the present study, the Km and Kcat values were determined to be 177±17 μM and 2.61±0.24 s^{-1} respectively (Table 2), in agreement with the previous study that reported the Km and Kcat values of recombinant hTDO of 190 μM and 2.1 s^{-1} , respectively, at pH 7.0 [43].

Table 2 Kinetic parameters of recombinant hTDO.

Enzyme	Kcat (s^{-1})	Km (μM)	Kcat /Km ($\mu M^{-1}. s^{-1}$)
hTDO	2.61±0.24	177±12	0.015

Kinetic parameters of recombinant hTDO in L-Trp oxidation. Mean and standard deviations from three replicate

experiments are shown.

2.3. Establishment of cellular assays of TDO activity

The A172 cell line has been reported as a model for cellular assay of TDO activity. However, A172 cells express all three Trp-metabolizing enzymes, IDO1, IDO2 and TDO (Fig. 2A). The catalysis by IDO1 and IDO2 in the kynurenine pathway would interfere with the experimental results. After screening several cell lines, we chose the U87 MG cell line, which uniquely expresses TDO (confirmed by Western blot, Fig. 2B and Fig. 2C), as our experimental model. The HEK293 cell line transiently transfected with the pcDNA3.1-hTDO plasmid (Fig. 2D) was used as an additional model of the cellular assay of TDO activity. In the present study, the IC_{50} values obtained in the two cell models are consistent indicating good reliability of these cellular assays.

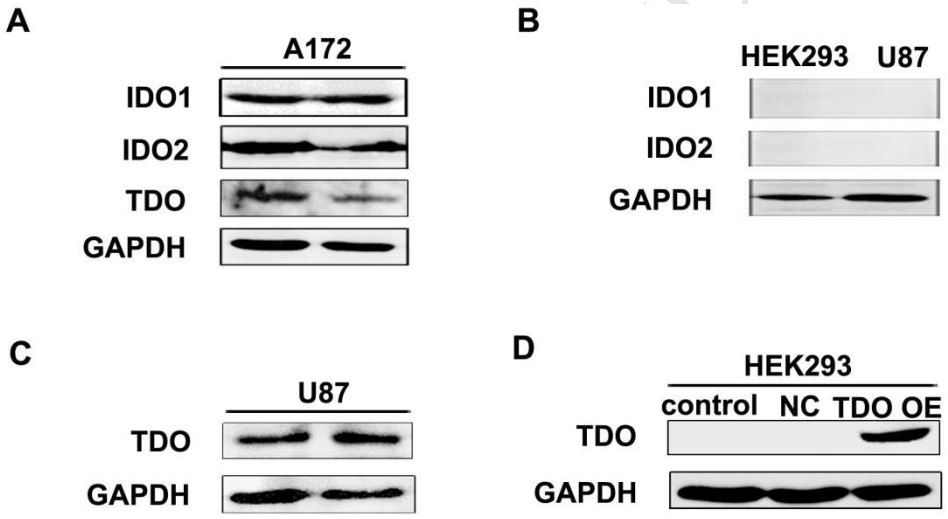


Fig. 2. Expression of IDO1, IDO2 and TDO in three cell lines confirmed by Western blot. A. Expression of IDO1, IDO2 and TDO in A172 cells. B. Expression of IDO1 and IDO2 in HEK293 and U87 MG cells. C. Expression of TDO in U87 MG cells. D. Expression of TDO in HEK293 cells (control), HEK293 cells transiently transfected with the pcDNA3.1 vector (NC), and HEK293 cells transiently transfected with the pcDNA3.1-hTDO plasmid (TDO OE).

Table 3 Primer sequences.

Recombinant plasmid	Primer sequence
pET28a-hTDO	F: 5'-CGCGGATCCGCGATGAGTGGGTGCCCATTTTTAGGA-3'
	R: 5'-CCGCTCGAGCGGTTAATCTGATTCCATCACTGCTG-3'

pcDNA3.1-hTDO	F:5'-TTTAAACTTAAGCTTGGTACCGCCACCATGAGTGGGTGCCCATTTTTAGGAA-3'
	R:5'-TCTAGACTCGAGCGGCCGCTTAATCTGATTCATCACTGCTGAAG-3'

Endonuclease sites are emphasized with a linear underline. Kozak sequence is emphasized with a wavy underline. pET28a-hTDO is digested with BamHI and XhoI. pcDNA3.1-hTDO is digested with Kpn I and Not I.

2.4. Enzymatic hTDO inhibitory activities of tryptanthrin derivatives

First, to validate our enzymatic assay of hTDO activity, the enzymatic hTDO inhibitory activities of IDO1 inhibitors L-1-MT (L-1-methyl tryptophan) and INCB024360 and the TDO inhibitor LM10 were tested. Our data showed that the IC_{50} value of INCB024360 against hTDO was 64.5 μM, which was consistent with the value of over 50 μM reported previously [44]. The Kyn production was increased with the addition of L-1-MT, suggesting that L-1-MT was a substrate rather than an inhibitor of TDO, which was consistent with the result reported in another article [45]. The IC_{50} and Ki values of LM10 were also consistent with previous studies [27].

Next, the potential hTDO inhibitory activities of tryptanthrin and its eight derivatives (**5a**-**5i**, Fig. 3) were evaluated. The structural features and representative NMR spectra of the tryptanthrin derivatives are shown in Fig. 3 and supplemental Fig S2, respectively. All compounds contained the same tryptanthrin skeleton, but the positions and chemical properties of the substituents were different. The enzymatic IC_{50}, Ki and type of inhibition were measured and are summarized in Table 4. All tested tryptanthrin derivatives possessed hTDO inhibitory activity (Fig. 4). Moreover, the majority of the compounds except **5g** and **5h** exhibited superior hTDO inhibitory effect than that of INCB024360 and the TDO inhibitor LM10. Compounds **5c**, **5d**, and **5i** were the most potent hTDO inhibitors with enzymatic IC_{50} at the nanomolar levels. Compounds **5b**, **5c**, **5d** and **5i** with the CH_3, F, Br or NO_2 substituents at 8-position displayed potent inhibition toward hTDO, demonstrating that the substituents at 8-position were beneficial for TDO inhibition. Moreover, the electron-withdrawing F, Br and NO_2 substituents were more effective than the electron donor CH_3 substituent. With an F substituent at 2-position, compound **5e** had weaker inhibitory effect on hTDO than compound **5a**, indicating that the substituent at 2-position was not favorable for the hTDO inhibitory activity. Therefore, compound **5f**, which possessed substituents at both 2-position and 8-position, had a lower inhibitory activity than **5b**, **5c**, **5d**, **5i** and a higher inhibitory effect than **5e**. In contrast to the compounds mentioned above, **5g** and **5h** showed much

lower inhibitory activities with the IC_{50} values of 44.8 μM and 244.2 μM, respectively, suggesting that the substituents at 7-position or 9-position have negative effects on hTDO inhibitory activity. Combined with our previous results on the IDO1 and IDO2 inhibitory activities of the same compounds [17, 46], the data indicate that these nine compounds displayed higher inhibitory activities toward IDO1 and TDO than that for IDO2. Compounds **5a**, **5c**, **5d**, **5f**, **5g**, **5h** and **5i** had comparable inhibitory activities toward IDO1 and TDO and were dual IDO1/TDO inhibitors. In contrast, compounds **5b** and **5e** were TDO-selective inhibitors showing a good selectivity over IDO1 (38-fold and 88-fold, respectively).

Then, the detailed kinetic analysis of these tyrptanthrin derivatives was performed based on plotting the reciprocal of the reaction velocity (1/V) against the inhibitor concentration ([I]). Despite having the same structural skeleton, the nine compounds exhibited different types of inhibition. Compounds **5a**, **5c**, **5d**, **5g** and **5i** were determined to be uncompetitive inhibitors, while compounds **5b**, **5e**, **5f** and **5h** were mixed-uncompetitive inhibitors. The Ki values were evaluated by plotting [S]/V against [I], where [S] represents the substrate concentration (Fig. 5). The Ki value of LM10 obtained in our study was 7.47 μM, which was consistent with the previous report (Ki=5.6 μM) [27]. The Ki values of most compounds except **5g** and **5h** were much lower than that of LM10.

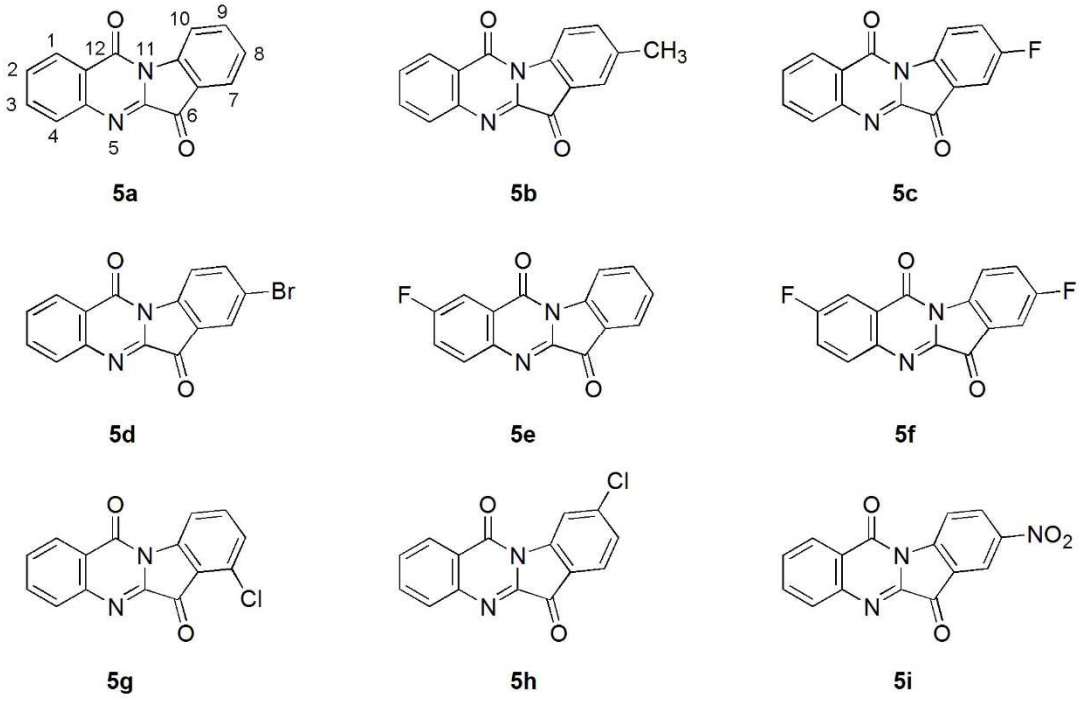


Fig. 3. Chemical structures of tryptanthrin derivatives with TDO inhibitory activities. The numbers indicate the

notion used throughout.

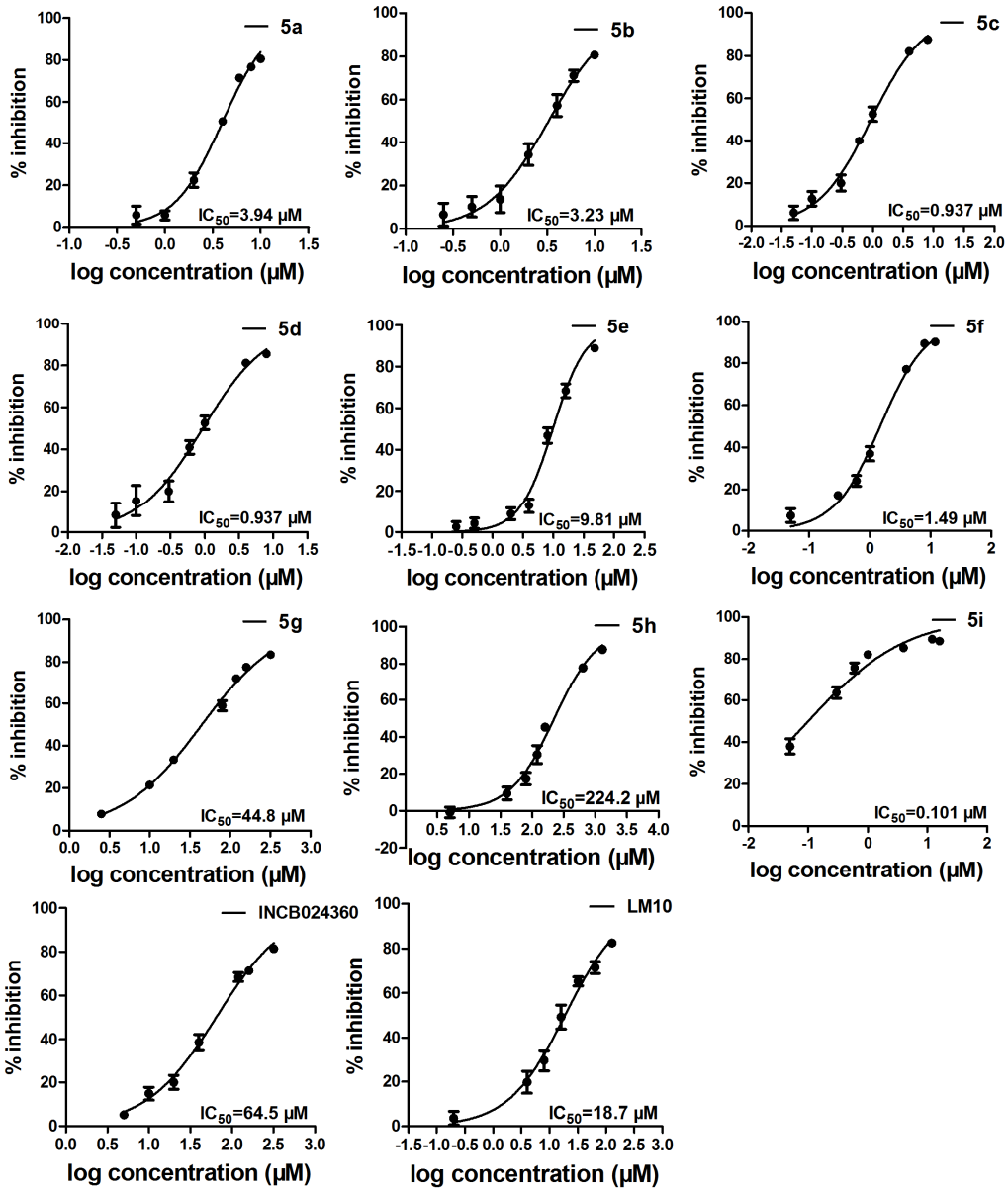


Fig. 4. Enzymatic hTDO inhibitory activities of the tryptanthrin derivatives, INCB024360 and LM10. The recombinant hTDO was incubated with different concentrations of the tryptanthrin derivatives. The percentage of inhibition was plotted against log[I] and the IC_{50} values were determined. The data for each curve were derived from triplicate experiments. Mean values are shown with error bars representing SEM. Data were evaluated using professional statistic software GraphPad Prism 5.0.

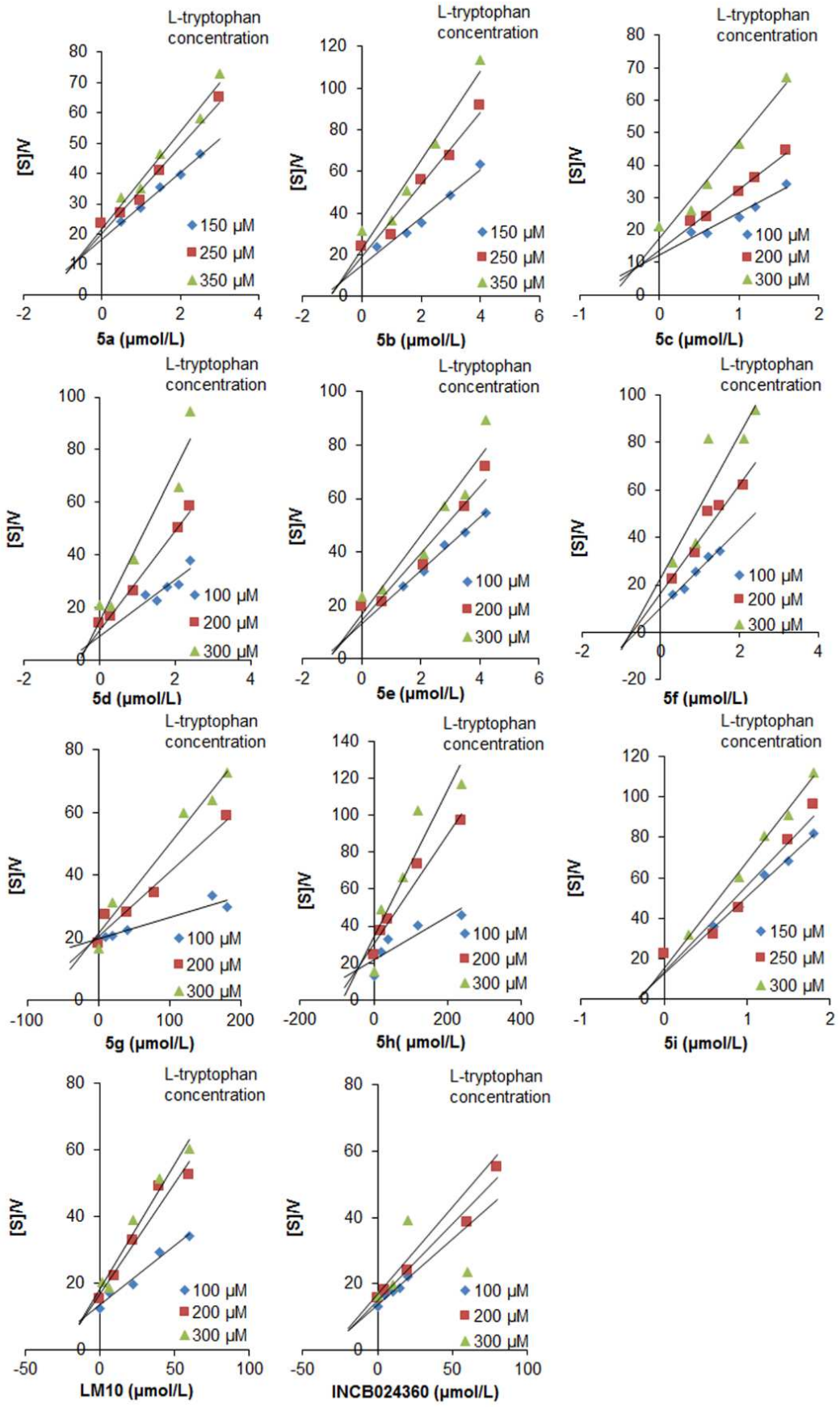


Fig. 5. Characterization of the tryptanthrin derivatives as potent hTDO inhibitors. Kinetic parameters of the nine

tryptanthrin derivatives, INCB024360 and LM10 were determined according to the plots of [S]/V against inhibitor concentrations [I]. The L-Trp concentrations varied from 100 to 350 μM. The intersection points in the plots were used to determine the Ki.

Table 4 Human TDO inhibitory activities of tryptanthrin derivatives.

Compound	Type of inhibition	Ki (μM)	Enzymatic IC_{50} (μM)	Cellular IC_{50} (μM)		
				U87 MG	HEK293-hTDO	Average
5a	uncompetitive	0.581 ± 0.107	3.94 ± 0.39	0.194 ± 0.083	0.140 ± 0.032	0.167
5b	mixed-uncompetitive	0.834 ± 0.107	3.23 ± 0.36	0.184 ± 0.054	0.215 ± 0.088	0.199
5c	uncompetitive	0.336 ± 0.014	0.937 ± 0.148	0.060 ± 0.009	0.068 ± 0.017	0.064
5d	uncompetitive	0.356 ± 0.078	0.937 ± 0.215	0.054 ± 0.015	0.053 ± 0.018	0.053
5e	mixed-uncompetitive	0.876 ± 0.040	9.81 ± 1.93	0.503 ± 0.050	0.241 ± 0.022	0.372
5f	mixed-uncompetitive	1.00 ± 0.08	1.49 ± 0.28	0.062 ± 0.014	0.059 ± 0.009	0.061
5g	uncompetitive	8.41 ± 2.34	44.8 ± 6.0	0.227 ± 0.080	0.229 ± 0.154	0.228
5h	mixed-uncompetitive	42.7 ± 4.7	224.2 ± 39.8	0.957 ± 0.191	0.733 ± 0.236	0.845
5i	uncompetitive	0.215 ± 0.020	0.101 ± 0.062	0.040 ± 0.011	0.061 ± 0.011	0.050
INCB024360	uncompetitive	21.7 ± 0.68	64.5 ± 8.7	0.260 ± 0.044	0.242 ± 0.086	0.251
LM10	uncompetitive	7.47 ± 2.81	18.7 ± 3.4	0.671 ± 0.142	0.398 ± 0.087	0.534

The numbers indicated the notation used throughout. All figures quoted were the mean ± SEM of triplicate measurements.

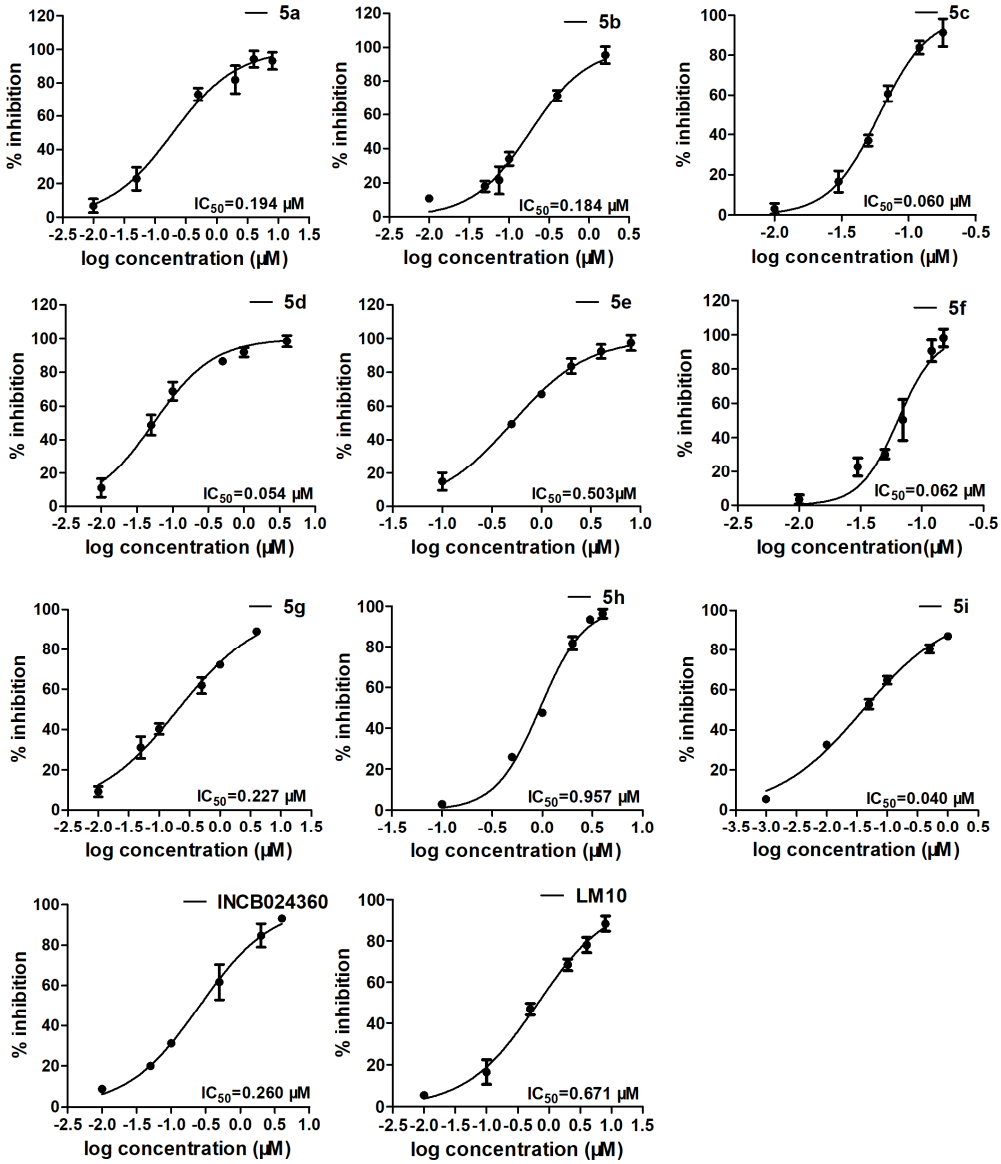
2.5. Cellular TDO inhibitory activities of tryptanthrin derivatives

The cellular TDO inhibitory activities of the nine compounds were evaluated by using TDO uniquely expressing U87 MG cells and pHEK293 cells transiently transfected with the pcDNA3.1-hTDO plasmid; the data were obtained in three replicate experiments (Table 4). The graphs of the IC_{50} values were processed by the GraphPad Prism 5.0 software (Fig. 6). The IC_{50} values calculated from the U87 MG assay were very close to those of the HEK293-hTDO assay. For instance, the IC_{50} of **5i** was 0.040 μM in the U87 MG assay and 0.061 μM in the HEK293-hTDO assay. Our results suggest that all compounds displayed TDO inhibitory activities at the cellular level. **5a**-**5g** and **5i** had higher activities than the TDO inhibitor LM10 (average IC_{50}

= 0.534 μM). Compounds **5c**, **5d**, **5f** and **5i** showed 3-4 times higher cellular inhibition activities than **5a**, demonstrating that the electron-withdrawing F, Br or NO_2 substituents at 8-position enhanced the TDO inhibitory activity of tryptanthrin (average IC_{50}=0.167 μM). Cellular inhibitory activities of compound **5b** was similar to that of **5a,** suggesting that the electron donor CH_3 substituent at 8-position was not as effective as the electron-withdrawing F, Br and NO_2 substituents. Compound **5e** displayed lower inhibitory activity than **5a,** demonstrating that the F substituent at 2-position had a negative effect on the TDO inhibitory activity of tryptanthrin. The TDO inhibitory activity of compound **5f** was lower than those of **5d** and **5i** because of the presence of the substituents at 2-position and 8-position. Compound **5g** was a TDO inhibitor with an IC_{50} value of 0.228 μM (average) suggesting that a substituent at 7-position is not favorable for TDO inhibition. Compound **5h** with a substituent at 9-position showed the lowest inhibitory efficacy among the nine compounds with an IC_{50} value of 0.845 μM, suggesting that a substituent at 9-position has a negative impact on the TDO inhibitory activity of tryptanthrin.

Taken together, these results from the enzymatic and cellular assays suggested that **5c**, **5d** and **5i** are potential TDO inhibitors worthy of further development.

A.


5a
IC50=0.194 μM
5b
IC50=0.184 μM
5c
IC50=0.060 μM
5d
IC50=0.054 μM
5e
IC50=0.503μM
5f
IC50=0.062 μM
5g
IC50=0.227 μM
5h
IC50=0.957 μM
5i
IC50=0.040 μM
INCB024360
IC50=0.260 μM
LM10
IC50=0.671 μM
% inhibition
log concentration (μM)
log concentration(μM)

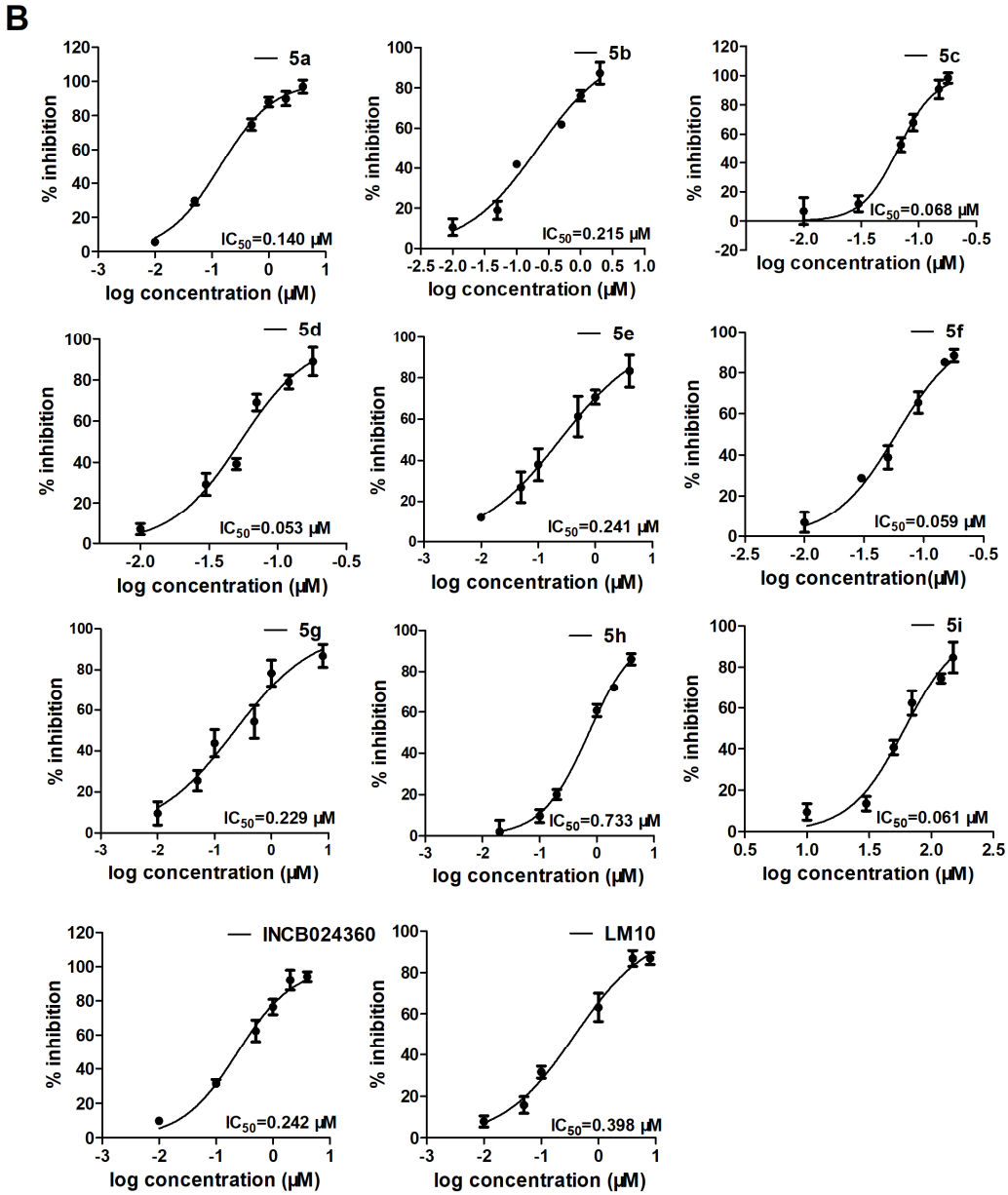


Fig. 6. Cellular TDO inhibitory activities of the tryptanthrin derivatives. The U87 MG cells (**A**) and HEK293 cells transiently transfected with the pcDNA3.1-hTDO plasmid (**B**) were treated with various concentrations of the tryptanthrin derivatives. The percentage of inhibition was plotted against log[I] and the IC_{50} values were determined. The data for each curve were derived from triplicate independent assays were presented. Mean values are shown with error bars representing SEM.

2.6. Molecular docking

To elucidate the binding modes of compounds **5c** and **5d** to hTDO, we performed molecular docking for compounds **5c** and **5d** using the crystal structure of hTDO (PDB ID 5TIA) and the program AutoDock 4.2 [47, 48]. Using the same docking parameters as in our previous study [49],

we carried out 200 independent docking runs and corresponding clustering analysis of the lowest-energy positions for each compounds and then obtained the lowest-energy position of a given compound in the active sites of hTDO.

As shown in Fig. 7, the lowest-energy positions of two compounds in the active site were almost the same suggesting that they possessed the same lowest-energy binding mode to hTDO. In this mode, the O atom at the position 6 of the tryptanthrin ring is directed toward the heme iron (Fe) by strong coordination interactions at an O-Fe distance of 2.3 Å. Hence, the whole compound (**5c** or **5d**) was in the active site pocket above the heme plane. Thus, the major driving forces of the compound binding to the active site were the O-Fe coordination interactions and the hydrophobic interactions between the compound and the hTDO active site. Because of the structural rigidity of the compounds, it appeared that the molecular size of the compounds also plays an important role in binding to the hTDO active site.

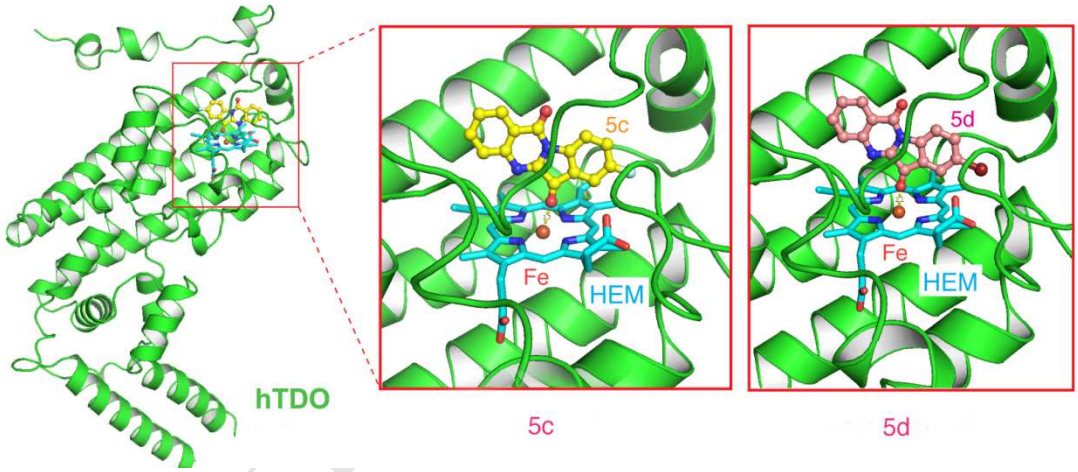


Fig. 7. Predicted binding modes of compounds **5c**, **5d** to the human TDO active site.

3. Conclusion

In this study, the induction conditions of recombinant hTDO and the pH conditions of the enzymatic assay of hTDO activity were optimized and the cellular assays of TDO activity were established. Nine tryptanthrin derivatives with various levels of TDO inhibitory activities were evaluated and found to bear different level of TDO inhibitory activities; most of the compounds exhibited higher TDO inhibitory activities than the previously reported TDO inhibitor LM10. Notably, compounds **5c**, **5d** and **5i** with electron-withdrawing substituents at 8-position displayed superior enzymatic and cellular TDO inhibitory potency among the nine compounds. Moreover,

molecular docking studies of **5c** and **5d** proved that the tryptanthrin ring is directly bound to the active site of hTDO through O-Fe coordination interaction and hydrophobic interactions. These results suggest that compounds **5c**, **5d** and **5i** are promising candidate molecules that might be developed into TDO inhibitors and applied in the TDO-related target therapy.

Our work will provide a basis for the design and screening of highly-efficient TDO inhibitors. Additionally, the inhibitors described here will assist in determining the physiological roles of TDO and its involvement in the pathology of tumor and neurological diseases.

4. Materials and methods

4.1. Materials

Most of the chemicals used in this study (L-Trp, L-ascorbate, catalase, methylene blue, and buffer components, Sigma-Aldrich) were of the highest analytical grade (97% purity), L-1-MT (Sigma-Aldrich, 95% purity), LM10 (Selleck, 99% purity) and INCB024360 (Selleck, 99% purity), were used without further purification. All tryptanthrin derivatives, if not mentioned specially, were dissolved in DMSO at a concentration of 10 mM. Details of the synthetic procedures used to prepare the candidate compounds and structural and analytical characterizations can be found in the corresponding article [46]. For the construction of the plasmids, a full-length hTDO cDNA fragment (*TDO2*, NCBI ID: NM_005651.3) was purchased from Youbio, primers for PCR amplification were ordered from Huagene company (China, Shanghai), restriction endonucleases were purchased from Takara, NovoRec one-step cloning kits were purchased from Novoprotein, DH5α competent *E. coli* cells were purchased from CWBIO, and culture medium for bacterial growth was purchased from Gibco. For protein purification, HisTrap 6FF Ni-NTA columns were purchased from Smart-life science, and HiLoad desalting columns and AKTA Purifier were from GE Healthcare. The human glioblastoma U87 MG cell line (ATCC number HTB-14), the human glioblastoma A172 cell line (ATCC number CRL-1620) and the human embryonic kidney HEK293 cell line (ATCC number CRL-1573) were purchased from the American Type Culture Collection. The transfection reagents Lipofectamine 2000 was purchased from Invitrogen Fetal bovine serum (FBS), Dulbecco's Modified Eagle's Medium (DMEM) and 1×opti-Minimal Essential Medium (MEM) were purchased from Gibco.

4.2. Construction of human TDO expression plasmids

To produce active human TDO, a pET28a-hTDO plasmid was constructed. For expression of hTDO in HEK293 cells, a pcDNA3.1-hTDO plasmid containing a Kozak sequence was constructed. A full-length hTDO cDNA fragment (*TDO2*, NCBI ID: NM_005651.3) was amplified by high fidelity polymerase chain reaction (PCR). The primer sequences for TDO amplification are shown in Table 3; the endonuclease sites are underlined with thick black lines. The hTDO cDNA was incorporated into the pET28a (+) (for *in vitro* expression) by introducing a BamHI site (GGATCC) and a XhoI site (CTCGAG) using T4 ligase (Takara) or into the pcDNA3.1(+) (for cellular expression) by introducing a Kpn I site and a Not I site using NovoRec one-step cloning kit according to the manufacturers' instructions (Novoprotein). The nucleotide sequences of all constructs were confirmed by sequencing at Huagene company. The pET28a-hTDO plasmid was used to transform DH5α and BL21 *E. coli* cells. The pcDNA3.1-hTDO plasmid was used to transform DH5α *E. coli* cells. The strains containing the hTDO constructs were stored in buffer containing 60% glycerol at -80□.

4.3. Expression of recombinant human TDO

A single kanamycin-resistant colony of BL21 *E. coli* cells transformed with the pET28a-hTDO plasmid (BamHI and XhoI) was grown overnight at 37□ in 100 mL of LB medium (pH=7.0) containing 50 μg/mL of kanamycin. A 20 mL aliquot was used to inoculate 1 L of LB medium. Bacteria were grown at 37□ with vigorous shaking at 220 rpm until the OD_{600} reached 0.6. Then, shaking was stopped, and the LB culture was cooled down to room temperature. To determine the effects of IPTG, ALA and hemin concentrations on recombinant hTDO activity, various concentrations of IPTG (0 mM, 0.2 mM, 0.5 mM, 0.8 mM, 1 mM) were added into the culture to induce the expression of hTDO, and various concentrations of ALA (0.2 mM, 0.5 mM) or hemin (0.01 mM, 0.5 mM) were added to increase the heme incorporation. The culture was grown at 23□ for 18 h with constant shaking at 180 rpm. Cells were harvested at 4,000 rpm for 15 min and freeze-thawed after overnight storage at -80□.

4.4. Purification of recombinant human TDO

The pelleted cells were resuspended in PBS (pH=8.0) and 1 mM of the protease inhibitor phenylmethylsulfonyl fluoride (PMSF) was added. Cells were homogenized in a low-temperature ultrahigh pressure continuous flow cell disrupter. Then, cell lysates were centrifuged at 13,000

rpm for 1 h at 4□ to remove the debris and obtain the supernatant. The supernatant of the cell lysates containing soluble recombinant hTDO was used for subsequent purification. We found that recombinant hTDO was difficult to elute when loaded on a prepacked Ni-NTA column; hence, we loaded the supernatant of the cell lysates on a gravity Ni-NTA column (6FF, Smart-life Sciences) that was pre-equilibrated and washed with PBS buffer (pH=8.0). Next, 20 mM and 40 mM imidazole buffers (NaCl, NaH_2PO_3, imidazole, pH=8.0) were used to remove the hybrid protein. Recombinant hTDO was harvested using the same buffer containing a higher imidazole concentration (250 mM). The efficiency of purification and specific activity (either crude or purified) of recombinant hTDO were measured.

To further purify recombinant hTDO and to remove the imidazole, preparative size exclusion chromatography (SEC) was used on a HiLoad16/600 Superdex 200 column which was preincubated with a mobile phase consisting of 25 mM Tris, pH 7.0, 1 mM EDTA, and 150 mM NaCl. Purified recombinant hTDO was concentrated in a 50 mL ultrafiltration concentrating tube. Concentration of recombinant hTDO was determined with a BCA kit (Beyotime) according to the manufacturer's instruction. Recombinant hTDO was used for the measurement of kinetic parameters. Recombinant hTDO was mixed with 60% glycerin at a proportion of 1:1 (v/v) for long term storage at -80□.

4.5. Enzymatic activity of recombinant human TDO

Determination of the values of kinetic parameters Km and Kcat of recombinant hTDO was performed by incubating recombinant hTDO with a series of concentrations of L-Trp (20 μM ~ 1600 μM) as the substrate. The Michaels-Menten equation was used to calculate the values of Km and Kcat, and the data were processed by GraphPad Prism 5.0 software.

4.6. Enzymatic assay of human TDO activity

The enzymatic assay of hTDO activity was carried out according to the method of Nicole Seegers et al with some modifications [34]. To evaluate the enzymatic hTDO inhibitory activities of tryptanthrins, purified recombinant hTDO (3 μg) was thawed at 37□ for 30 s, and added to a 37□ pre-equilibrated incubation system (500 μL) containing L-Trp (400 μM), ascorbate (10 mM), methylene blue (20 μM), bovine liver catalase (200 μg/L) and 50 mM potassium phosphate buffer (pH=7.0); these components functioned as the medium that maintained the reducing environment.

The compounds (2 μM for preliminary screening and appropriate concentration gradients for the Ki and IC_{50} values) were added to this mixture and reacted at 37□ for 30 min in a thermostated water bath. The reaction was terminated by addition of 200 μL of trichloroacetic acid (TCA) and the tubes were incubated at 65□ for 15 min to promote Kyn production. The 100 μL supernatant from each tube was mixed with an equal volume of dimethylaminobenzaldehyde (DMAB) in acetic acid (3‰, w/v) and the time-dependent formation of L-Kyn was monitored at 492 nm wavelength with a Multiscan spectrum Mk3 (Thermo Fisher). Each reaction was conducted in triplicate. In the assays, controls were included to eliminate interference including any reactions of the inhibitor molecules with the substrate or the substrate with DMAB in the absence of TDO. The enzymatic hTDO inhibitory activities of the IDO1 inhibitors INCB024360 and L-1-MT and the TDO inhibitor LM10 were determined to confirm the efficiency of our assay. The data were processed with the GraphPad Prism 5.0 software.

4.7. Determination of the inhibition type and kinetic parameters

In this study, we further evaluated the enzymatic hTDO inhibitory activities and inhibition characteristics of the tryptanthrin derivatives. The Ki values were determined using the improved version of the Dixon plot by plotting [S]/V against [I], where [S], V and [I] represent substrate concentration, reaction velocity and inhibitor concentration, respectively, and the substrate concentrations varied from 100 μM ~ 350 μM. The intersection point of three different L-Trp concentration trendlines was calculated as the Ki value. The types of inhibition were determined by plotting 1/V against [I]; the concentrations of inhibitors varied over a two-fold range above and below the concentration corresponding to approximately a half of one hundred percent inhibitory activity.

4.8. Cellular assay of TDO activity

We have established the assays to accurately measure the cellular TDO inhibitory activities of the nine compounds.The Kozak sequence-containing pcDNA3.1-hTDO plasmid was transiently transfected into the HEK293 cells and TDO uniquely expressing U87 MG cells were cultured in DMEM medium (Gibco, Life Technology) supplemented with 10% FBS (Gbico, Life Technology), 50 U/mL of penicillin and 50 mg/mL of streptomycin at 37□ at 5% CO_2 and 95% humidity. The compounds were diluted in DMSO to the concentration of 500 μM and then further diluted in

DMEM; the final DMSO concentration in the system was less than 1% (w/v). Cells (2×10^4 per well in 10 μL) were seeded in a 96-well cell culture plate (Nunc) and allowed to adhere by incubation for 12 h. Then, the incubation continued for 8 h after addition of a serial dilutions of the tested compounds and L-Trp (200 μM, sterilized by filtration). DMEM medium supernatant (140 μL) was transferred to a new tube and the reaction was terminated by addition of 10 μL TCA (30%, w/v). Then, the tubes were transferred into a 65□ water bath for 15 min to facilitate the N-formylkynurenine to L-kynurenine transition. Then the tubes were centrifuged at 13,300 rpm for 10 min .The supernatant (100 μL) was transferred to a new 96-well microplate and mixed with an equal volume of DMAB dissolved in acetic acid (3‰, w/v). Finally, the absorbance (corresponding to detection of the L-Kyn-DMAB adduct) was measured at 492 nm wavelength. Cellular IC_{50} values were determined via nonlinear regression analysis using the GraphPad Prism 5.0 software.

4.9. Western blot analysis of the expression of IDO1, IDO2 and TDO

HEK293 cells, HEK293 cells transiently transfected with the pcDNA3.1(+) vector, HEK293 cells transiently transfected with the pcDNA3.1-hTDO plasmid, U87 MG cells and A172 cells were harvested and lysed on ice for half an hour. Western blot assay was performed to analyze the expression of IDO1, IDO2 and TDO using a standard procedure. Anti-TDO (Proteintech, Cat. No. 15880-1-AP), anti-IDO1 (CST, Cat. No. 68572), anti-IDO2 (Santa, Cat. No. sc-87164) and anti-GAPDH (Beyotime, Cat. No. AG019) antibodies were added in a primary antibody dilution buffer (Beyotime) and allowed to incubate overnight at 4□. Then, the membranes were washed with PBST (PBS containing 3‰ Tween-20) 3 times (5 min, 10 min, 15 min) before the secondary antibodies (Beyotime, goat anti-rabbit second antibody, goat anti-mouse second antibody) were added and the incubation continued at room temperature for 1 h. Then the membranes were washed with PBST 3 times (10 min each time). High-signal ECL Western blotting substrate (Tanon) was added and the membrane was immediately imaged using a chemi scope (clinx).

4.10. Molecular docking

The method described here was previously reported by Huang et al [49]. The AutoDock program (version 4.2) with the Lamarckian genetic algorithm was used for the molecular docking to predict the binding modes of compounds **5c** and **5d** to hTDO. Pretreatment of the compounds

and the hTDO receptor structure for docking was carried out with the AutoDock Tools program suite (see http://mgltools.scripps.edu). Two hundred independent docking runs were conducted for each compound. To perform the Lamarckian genetic algorithm, a population of 150 random ligand conformations in random orientations and at random translations was first generated and the population evolved according to the algorithm. The process was terminated after 27,000 generations and a maximum of 1,500,000 energy evaluations. Other parameters for running the AutoDock program were set to the default values.

Author contributions

S. Z. performed most of the enzymatic, cellular, and related experiments. F. Q., X. F., D. Y., Q. H, and H. H. helped with resolving experimental technical problems and data analysis. C. K. synthesized tryptanthrins and analyzed chemistry and structure-related results. Q. Y. initiated the research, led the project team, designed experiments, analyzed the data, and wrote the manuscript.

Disclosure

There is no interest to declare. All authors have approved the final manuscript.

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Abbreviation used

TDO: Tryptophan 2,3-dioxygenase; Ki: inhibitory constant, IC_{50}: half maximal inhibitory concentration; Trp: Tryptophan, NAD: nicotinamide adenine dinucleotide; Kyn: kynurenine; QUIN: quinolinic acid; IDO1: indoleamine 2,3-dioxygenase 1; IDO2: indoleamine 2,3-dioxygenase 2; AD: Alzheimer's disease; AhR: aryl hydrocarbon receptor; PD: Parkinson's disease; HPLC: high-performance liquid chromatography; hTDO: human TDO; ALA: 5-aminolevulinic acid; IPTG: isopropyl β-D-1-thiogalactopyranoside; SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis; MW: molecular weight; LB: Luria-Bertani; 1/V: the reciprocal of the reaction velocity; [I]: inhibitor concentration; [S]: substrate

concentration; PDB: protein data bank; FBS: fetal bovine serum; DMEM: Dulbecco's Modified Eagle's Medium; MEM: Minimal Essential Medium; PCR: polymerase chain reaction; PBS: phosphate buffer saline; PMSF: phenylmethylsulfonyl fluoride; EDTA: ethylene diamine tetraacetic acid; SEC: size exclusion chromatography; TCA: trichloroacetic acid; DMAB: 4-dimethylaminobenzaldehyde; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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Highlights:

> New cellular assays of TDO activity are established.

> Tryptanthrin and its derivatives are potential TDO inhibitors.

> Molecular docking studies suggest **5c** and **5d** bind to the active site of hTDO.