Research paper

Establishment of a human indoleamine 2, 3-dioxygenase 2 (hIDO2) bioassay system and discovery of tryptanthrin derivatives as potent hIDO2 inhibitors

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ABSTRACT

As an analogue of IDO1 (indoleamine 2, 3-dioxygenase 1), the well-known new therapeutic target, IDO2 is receiving increased attention. Herein, the expression and purification of recombinant human IDO2 (hIDO2) and the establishment of a hIDO2 bioassay system on both enzymatic and cellular levels are described. Nine tryptanthrin derivatives were screened for potential hIDO2 inhibitory activities, and their Ki values, enzymatic and cellular IC50 values, as well as the types of inhibition were measured. The tryptanthrin derivatives 5i, 5c and 5d (especially 5i) were found to be potent hIDO2 inhibitors with superior efficiency far better than that of the most frequently-used inhibitor L-1-MT. Two ultimate purposes of the present study have been achieved: establishing an IDO2 bioassay system and screening novel IDO2 inhibitors that can be used (directly or with some modifications) for potential therapeutic applications.

1. Introduction

The kynurenine pathway is responsible for approximately 99% of the oxidative cleavage the least abundant essential amino acid, tryptophan (Trp) [1,2]. The latest discovered indoleamine 2,3-dioxygenase-2 (IDO2) along with indoleamine 2,3-dioxygenase 1 (IDO1) [3,4] and tryptophan 2,3-dioxygenase (TDO) [5,6] are all capable of independently catalyzing the initial and rate-limiting step of tryptophan degradation along the kynurenine pathway to generate a series of down-stream metabolites such as quinolinic acid and kynurenic acid in mammals. As a heme-containing extracellular and intracellular monomeric enzyme, IDO1 is widely expressed in many organs, tissues and cell types [7–10]. Due to its indispensable immunomodulatory roles in pregnancy [11,12], cancer [13], allergy [14] and the proverbial central nervous system disorders Alzheimer’s disease [15], IDO1 has emerged as a significant new therapeutic target.

However, in contrast to the thorough studies on IDO1, the physiological and pathological roles of IDO2 have, to a large extent, remained unclear. In both human and mice, the encoding gene of IDO2 (Ido2) resides in the adjoining downstream of IDO1 gene (Ido1) on the chromosome 8 [16–18]. IDO2 displays much more conserved distribution characteristic than that of IDO1 as IDO2 protein exists in various lower vertebrates including platypus, frogs, zebrafish and chicken. The last three species reveal only one
IDO-like protein, extremely similar to mammalian IDO2, which offers us a good reason to believe that the IDO1 encoding sequences may have been derived from an ancestor IDO2-like gene duplication [18–20] occurring before the divergence of vertebrates [21]. IDO2 is primarily expressed in liver, kidney tubules and reproductive tracts (e.g. epididymis, spermatozoa) apart from various cell lines such as pancreatic, gastric, colon and kidney carcinoma [16,17]. IDO2 is uniquely regulated by AhR (Aryl hydrocarbon receptor) and involved in tumorigenesis [22, 23].

There are two broadly distributed single-nucleotide polymorphisms (SNPs) in the coding region of the human IDO2 gene, namely R248W and Y359 Stop, which respectively attenuate and abolish the catalytic activity of IDO2 [16]. These SNPs exert great influence on IDO2-specific spontaneous immune responses and IDO2-based anti-cancer vaccination [24], act as biomarkers for therapeutic responses [25] and may therefore affect an individual’s ability to respond to the drugs targeting IDO2. Previous study showed that IDO2 was critical for IDO1-mediated T-cell regulation ability to respond to the drugs targeting IDO2. Previous study showed that IDO2 was critical for IDO1-mediated T-cell regulation and inflammatory responses in IDO2−/− mice [26]. It was found that in human T cell proliferation and immunoreacations, IDO2 could notably suppress T cell growth, and the suppression could not be reversed or eliminated by either tryptophan or D-/L-1-MT (D- or L-1-methyl-tryptophan), which was inconsistent with IDO1 [27]. Concerning whether the D- or L-1-MT is a more potent inhibitor of IDO2, so far, no general consensus has been reached [19, 20, 28–31].

There are also limited reports on the preparation of recombinant IDO2 and its in vitro activity assay. Metz et al. cloned IDO2 on the basis of partial IDO1 structural homologies that were found downstream of the human IDO1 gene in a region of chromosome 8p12 [16]. The previous studies showed that both human and mouse IDO2 were less active than IDO1, and the mouse IDO2 exhibited higher catalytic activity compared to human IDO2 [16,23,32]. Therefore, it is in desperate need to express and purify recombinant IDO2 protein as well as to establish an IDO2 bioassay system.

There has always been a continuous demand for the development of selective IDO2 inhibitors, which will provide valuable tools for investigating the functions of IDO2 in both normal physiology and tumor immune evasion. However, only few compounds are known to be IDO2 inhibitors, and the well-known compound of D-1-MT, the former chemotherapeutic agent used in clinical trials, is found to be barely valid in the subsequent reports [20, 27, 28, 31]. By screening a library of FDA-approved drugs in the HEK293T cells transfected by mouse IDO2 gene, the proton pump drug tenatethrin (a natural product from the Chinese medicinal plants) was found to be an efficacious miDO2 inhibitor (with a IC50 value of 1.8 μM), but not for IDO1 or TDO inhibition [33]. For the last several years our group has been dedicated to developing novel potent human IDO1 and IDO2 inhibitors, which cover the isolation of new IDO1 inhibitors from natural medicines with therapeutic potential in Alzheimer’s disease or cancer, the design and synthesis of the novel IDO1 inhibitor skeletal structures [34–38]. Our studies on these IDO1 inhibitors have certainly benefited to the development of promising IDO2 inhibitors.

2. Results and discussion

2.1. Purification of active recombinant hIDO2

It is reported that the two proteins of IDO1 and IDO2 share 43% similarity of their amino acid sequences in both human and mouse [17,18]. The recombinant IDO2 especially human IDO2 has been the more challenging enzyme to demonstrate activity ever since its discovery, although the purification procedures and activity assays of recombinant human or mouse IDO1 proteins have been well established. The full-length transcript of human IDO2 gene [16] has been found to be inactive, while the alternate transcript starting at the second Met and excluding the 13 NH2 terminal amino acids has been shown to encode functionally active IDO2 [17,39]. Thus, in the present study, a recombinant vector pET28a-hIDO2 containing truncated IDO2 encoding sequence (exclude the 5’ terminal 39 bases) was constructed (for detailed nucleotide and amino acid sequences of hIDO2 constructs used in this study, see Supplemental Fig. S1). In addition, careful operation is crucial for the preservation of the activity of recombinant IDO2 because IDO2 has been reported to be more sensitive to buffer conditions (such as pH) than IDO1 [17,20]. Therefore, the samples were flash-frozen in liquid nitrogen and stored at −80 °C after being eluted from affinity chromatography Ni-NTA columns and molecular sieve columns. As shown in the SDS-PAGE (Fig. 1A), after induced by IPTG, a distinct and over-expressed band at ~45 kDa of human IDO2 was present in the samples of total protein (lane 3) and cell lysate supernatant (lane 4), and after eluted from a Ni-NTA chelating column with 250 mM imidazole, only one specific band with the same MW was observed (lanes 6–7), indicating the high purity of an isolated recombinant hIDO2 protein. The western blot shown in Fig. 1B indicated that the purified sample had a protein band of ~45 kDa, the same as predicted molecular weight (MW).

To determine the efficiency of our purification, the specific activity of hIDO2 enzyme was measured. The data in Table 1 shows a summary of purification of recombinant hIDO2 from the bacterial pellets of 2 L of culture. The hIDO2 enzyme was purified approximately 16.8-fold from its crude extract through the chromatography step using metal affinity chromatography (Ni−NTA agarose) and the yield was 15.2 mg/L of LB broth.

2.2. Enzymatic activity of recombinant hIDO2

The kinetic parameters for recombinant mouse IDO2 have been well studied and reported to be Kcat of 98.7 ± 8.6 min−1, Km of 45,900 ± 5800 μM and the Kcat/Km of 2.15 × 10−3 μM−1 min−1 [20,21]. To determine the enzymatic parameters (kcat and Km) of recombinant hIDO2, reactions were performed at the optimal pH 7.5 using L-Trp as substrate, and the data are listed in Table 2.

The Km value, Kcat value and the ratio Kcat/Km of hIDO2 were determined to be 9360 ± 810 μM, 5.62 ± 0.67 min−1 and 6 × 10−4 μM−1 min−1, respectively. Our data are comparable to those for recombinant human IDO2 reported by G. Pantouris et al. [30]. The hIDO2 showed much lower affinity for L-Trp and lower catalytic efficiency compared to hIDO1 (Km value 14.1 ± 0.44 μM, Kcat value 60 ± 4.8 min−1 [39]). While compared to the kinetic parameters of miDO2 [20,21], hIDO2 showed higher affinity for the substrate L-Trp (Km), but lower catalytic efficiency (Kcat and Kcat/ Km). In general, the recombinant hIDO2 prepared in this study bore comparable catalytic efficiency as that reported by G. Pantouris et al. [30].

2.3. Enzymatic hIDO2 inhibitory activities of tryptanthrins

IDO1, an isoform of IDO2, has emerged as a promising drug target due to its vital roles in tumor genesis and immune tolerance and chemotherapy of various cancers [13]. Different from IDO1, IDO2 shows relatively less efficiency in catalyzing tryptophan degradation and bears characteristics quite distinct from that of IDO1 in terms of substrate specificity and affinity [30]. Moreover, the biochemical characteristic of IDO2 has not been fully elucidated due to the lack of an appropriate in vitro bioassay. Using the prepared recombinant hIDO2, an in vitro IDO2 bioassay system was established, and the potential IDO2 inhibitory activities of tryptanthrin (a natural product from the Chinese medicinal plants
Polygonum tinctorium and Isatis tinctoria) and its derivatives, in comparison with L- or D-1-MT were evaluated. The structure features and representative NMR spectra of tryptanthrin derivatives were shown in Fig. 2 and Supplemental Fig. S3 respectively. The synthetic procedures and conditions of these compounds were described in our previous study[38].

To obtain the kinetics parameters of their Ki and IC50 values, tryptanthrin and its eight synthesized derivatives along with 1-MT isomers were subjected to hIDO2 inhibition assays. The IC50 values in Table 3 showed that all nine tryptanthrin compounds displayed hIDO2 inhibitory activities, especially, the compounds 5c, 5d and 5i demonstrated much stronger inhibition (7.68 μM, 8.26 μM and 1.87 μM) than both L-1-MT (82.53 μM) and D-1-MT (262.75 μM). Based on our data of both the IC50 and Ki values shown in Table 3, L-1-MT was a more potent IDO2 inhibitor than D-1-MT, which is consistent with the previous reports [19, 20, 28, 29, 31]. Specifically, compared to that of D-1-MT (former cancer immunotherapy adjuvant in clinical trials), the IC50 value of 5i was 140.5-fold more, 5c and 5d were 34.2 and 31.8-fold more, 5e and 5h were 4.6 and 3.9-fold more inhibitory effect. Thus, all these nine tryptanthrins were confirmed as potent human IDO2 inhibitors, and also reversible inhibitors when the reaction velocity (V) against the enzyme amount ([E]) was plotted (data not shown). Furthermore, their kinetic constant Ki values were assessed by plotting [S]/V against inhibitor concentration, where [S] represented the substrate concentration (Fig. 3). The Ki value of the most promising inhibitor candidate 5i was in a nanomolar level (0.97 μM), 5c and 5d were 3.51 μM and 5.62 μM, respectively, while under the same conditions, the Ki value of L-1-MT was 425 μM, and the Ki value of D-1-MT was greater than 3900 μM with no specific number detected (Table 3). In the other word, the tryptanthrin derivatives 5i, 5c and 5d showed 438.1-, 121.1- and 75.6-fold more potent than that of L-1-MT.

Next, the kinetics parameters of L-1-MT and these nine tryptanthrins were determined according to the plots of [S]/V against inhibitor concentrations. As shown in Fig. 3, the kinetic graphical modes of seven tryptanthrins (5a, 5c, 5d, 5e, 5g, 5h and 5i) of the nine fitted in an uncompetitive inhibition type, while 5b and 5f fitted in a noncompetitive and mixed-competitive type. As expected, the most commonly used IDO1 inhibitor L-1-MT exhibited a competitive type.

It is worth mentioning that the Ki value of L-1-MT obtained in this study (425 μM, data with good repeatability) is comparable to the findings of Pantouris et al. in which the Ki values for L- and D-1-MT were 97.6 μM and 37.8 μM, respectively [39].
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Table 3
Human IDO2 inhibitory activity of tryptanthrin derivatives.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Type of inhibition</th>
<th>K_i (μM)</th>
<th>IC_{50} (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-L-MT</td>
<td>Competitive</td>
<td>425 ± 53</td>
<td>82.5 ± 40</td>
</tr>
<tr>
<td>1-D-MT</td>
<td>Competitive</td>
<td>ND^a</td>
<td>262.7 ± 39</td>
</tr>
<tr>
<td>5a</td>
<td>Uncompetitive</td>
<td>15.2 ± 2.7</td>
<td>17.3 ± 3.6</td>
</tr>
<tr>
<td>5b</td>
<td>Noncompetitive</td>
<td>34.8 ± 4.3</td>
<td>32 ± 0.7</td>
</tr>
<tr>
<td>5c</td>
<td>Uncompetitive</td>
<td>3.5 ± 0.9</td>
<td>7.6 ± 1.5</td>
</tr>
<tr>
<td>5d</td>
<td>Uncompetitive</td>
<td>5.6 ± 0.4</td>
<td>8.3 ± 2.3</td>
</tr>
<tr>
<td>5e</td>
<td>Uncompetitive</td>
<td>105.1 ± 25</td>
<td>56.8 ± 5.4</td>
</tr>
<tr>
<td>5f</td>
<td>Mixed competitive</td>
<td>13.2 ± 1.6</td>
<td>21.7 ± 2.9</td>
</tr>
<tr>
<td>5g</td>
<td>Uncompetitive</td>
<td>24.6 ± 4.4</td>
<td>43.4 ± 7.8</td>
</tr>
<tr>
<td>5h</td>
<td>Uncompetitive</td>
<td>39.5 ± 1.2</td>
<td>67.9 ± 6.1</td>
</tr>
<tr>
<td>5i</td>
<td>Uncompetitive</td>
<td>0.97 ± 0.1</td>
<td>1.8 ± 0.6</td>
</tr>
</tbody>
</table>

The numbers indicate the notation used throughout. All figures quoted are the mean ± SEM of 3 independent replicate measurements.

^a Not detected.

MT were 300 ± 25 and 3300 ± 400 μM [30], indicating that an enzymatic activity reaction system is successfully established and the data obtained in this study are solid.

2.4. Cellular hIDO2 inhibitory activities of tryptanthrins

Developing the cell-based assays of IDO2 is necessary in order to further evaluate the IDO2 inhibitory potential of these tryptanthrins. It is well known that cellular assays are of great importance in the drug design processes and the assay results become necessary references to any promising drug candidates prior to their clinical trials. It has been reported that IDO2 is expressed in mammalian cells and the activity of IDO2 has been blocked by 1-MT [30], indicating that an enzymatic activity reaction system is successfully established and the data obtained in this study are solid.

Before measuring the inhibitory activities of tryptanthrins, the amount of kynurenine released from the cells was spectrophotometrically measured, and to find that pcDNA3.1(+) hIDO2 transfection in combination with addition of 200 μM L-Trp in cell culture medium did increase kynurenine production (Supplemental Fig. S2), and subsequent cellular assays were taken on this basis. Each graph of IC_{50} values showed the data averaged from three separate experiments and expressed as the mean ± SEM with four replicates within each experiment. As shown in Fig. 5 and Table 3 the IC_{50} value of tryptanthrin derivative 5i was 0.446 μM, exhibiting 185- and 333.8-fold higher cellular inhibitory activity of hIDO2 than that of L- and D-1-MT, while the cellular IC_{50} values of 5c, 5d, 5a and 5f were less than 10 μM, exerting 121.1-, 46.2-, 29.7- and 18.2-fold higher cellular inhibitory activity than D-1-MT, respectively. All the other tryptanthrin derivatives displayed comparable inhibitory activities against hIDO2 in the cellular assays, even the least potent compound 5e showed ~1.5-fold more inhibitory effect than L-1-MT and 3.94-fold more effective than D-1-MT.

Taken together, these results of in vitro and cellular assays led us to propose that tryptanthrin derivatives 5i along with 5c and 5d were promising small molecule drug candidates that might be developed into immunotherapeutic agent to restrain tumor immunoescaping.

3. Conclusion

In summary, we purified recombinant hIDO2 and characterized its kinetic properties in this study. Our data demonstrated that all nine tested tryptanthrins were potent hIDO2 inhibitors which significantly reduced IDO2 activity, and the strongest inhibitor was 5i with K_i value of 0.97 μM. Combining with our previous results of the IDO1 inhibitory activities of the same compounds [38], we conclude that these tryptanthrins bear the inhibitory activities of both IDO1 and IDO2, but with much stronger inhibitory potency on IDO1 than that on IDO2 (Supplemental Fig. S4).

The inhibitors described here will assist in determining the physiological roles of these two enzymes (IDO1 and IDO2) and their involvement in tumor immune tolerance. Therefore, our findings would provide significant clues for the design and development of human IDO-related drugs. Hopefully, in the near future, in conjunction with pre-existing therapies, these tryptanthrin
Characterization of tryptanthrins as potent hIDO2 inhibitors. Kinetics parameters of L-1-MT and nine tryptanthrins were determined according to the plots of \([S]/V\) against inhibitor concentrations \([I]\). The L-tryptophan concentration varied from 20 to 40 mM. Points of intersection in the plots were used for determining the kinetic constant \(K_i\).
inhibitors will be widely employed in immunotherapy for the treatment of tumors, Alzheimer's disease, neurological disorders and other serious diseases featuring pathological tryptophan metabolism.

4. Materials and methods

4.1. Materials

Most of the chemicals used (L-Trp, L-ascorbic acid, methylene blue, catalase etc.) and those for buffers were of the highest analytical grade (97% purity), D/L-1-methyl-tryptophan (95% purity) and used without further purification. Unless otherwise stated, all reagents were obtained from Sigma-Aldrich. All tryptanthrin derivatives, if not mentioned specially, were dissolved in DMSO making a concentration of 10 mM. For protein purification, HisTrap 6FF columns, HiLoad desalting columns and AKTA Purifier were from GE Healthcare.

Human glioblastoma U87 MG cells (ATCC Number: HTB-14) were purchased from the American Type Culture Collection. For cell culture and transfection, DMEM (Dulbecco's Modified Eagle's Medium) and Lipofectamine 2000 were from Invitrogen, while opti-MEM and FBS (fetal bovine serum) were from Gibco and Hyclone, respectively.

Details of synthetic protocols used to prepare the candidate compounds as well as structural and analytical characterizations can be found in corresponding literature [38].

4.2. Construction of expression vectors

In order to produce active human IDO2, the Escherichia coli expression recombinant plasmid pET28a-hIDO2 without the first 13 amino acids of the human IDO2 [39] was constructed, while the genes for residues from 14 to 420 amino acids were fused to a C-terminal His(6) tag.

The full length and shortened version of human IDO2 cDNA were synthesized by Shanghai GenePharma Co. based on the predicted human IDO2 sequences (Ido2, NCBI ID: NM_194294.2). The synthesized cDNA fragment was then amplified by high fidelity PCR system using the following primers shown in Table 4, in which endonuclease sites were underlined. Vectors pET28a and pcDNA3.1(+) were cut by Nco I/Xho I and Kpn I/Nco I (TakaRa), respectively. The amplified cDNA product was purified and directionally recombined into digested pET28a (for in vitro expression) or pcDNA3.1(+) (for cellular expression) using NovoRec one-step cloning kits according to the manufacturer's instructions (Novo-protein). The nucleotide sequences of all constructs were confirmed by sequencing.

4.3. Expression of hIDO2

Starter cultures of transformed Escherichia coli BL21(DE3) (Agilent Technologies) were grown overnight in 20 mL of Luria-Bertani broth (LB) medium containing kanamycin (50 µg/mL) at 37 °C, and scaled up to 1 L LB/kanamycin media. When a cell density of ~0.6 O.D. at 600 nm was reached, the cultures were cooled down to room temperature. Soluble hIDO2-His(6) production was induced by addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.05 mM. To maximize heme incorporation, the culture media were supplemented with 0.5 mM δ-aminolevulinic acid (ALA, a natural precursor of heme) solution per flask, and grown overnight at room temperature to a final cell density of ~9 O.D. at 600 nm. Cells were harvested by centrifugation at 4000 × g, 4 °C for 30 min, collected pellets were stored at −20 °C or resuspended and homogenized in 25 mM Tris buffer (pH 7.5, containing 150 mM NaCl) for subsequent experiments.

4.4. Purification of hIDO2

Collected bacterial cultures of IDO2 were resuspended in ice-cold 25 mM Tris–HCl buffer (pH 7.5), containing 150 mM NaCl, 10 mM imidazole, 1 mM phenylmethylsulphonyl fluoride (PMSF). The bacterial suspension was incubated with 1 mg/mL hen egg white lysozyme on ice for 30 min. Lysis was completed in high pressure cell cracker at 4°C before centrifugation at 13,000 × g, 4 °C for 1 h to remove cell debris and obtain supernatant. The cell lysate was then applied to a 5 mL HisTrap 6FF chelating column which was pre-equilibrated and washed with the basal buffer (25 mM Tris, pH 7.5, 150 mM NaCl, 20 mM imidazole). Following this step, IDO2 was eluted using the same buffer but with a higher imidazole concentration (250 mM). The efficiency of purification by Ni-NTA and the specific activity of hIDO2 (either crude or purified) were measured according to the protocols reported by Austin et al. [40] with minor modifications (pH 7.5, 40 mM L-tryptophan as substrate).

In order to further purify hIDO2 and remove imidazole, preparative size exclusion chromatography (SEC) was used on a HiLoad 16/600 Superdex 200 column which was pre-incubated with a mobile phase consisting of 25 mM Tris, pH 7.5, 1 mM EDTA, 150 mM NaCl. Following this step, obtained fusion protein was concentrated through application of 15 mL ultrafiltration device (Millipore). All purification steps were performed at 4 °C on an Akta Purifier chromatography system. Protein concentration was determined with BCA kits according to the manufacturer’s instructions (Bio-time). The purified recombinant hIDO2 was used for subsequent kinetic assays or diluted with a 1:1 addition of 60% glycerol for storage at −80 °C, flash-frozen in liquid nitrogen to preserve enzyme activity.
Fig. 5. Cellular hIDO2 inhibitory activities of tryptanthrins. The U87 MG cells transiently transfected by recombinant vector pcDNA3.1(+)–hIDO2 were subjected to different concentrations of tryptanthrins. The percentage of inhibition against log [I] was plotted and IC₅₀ values were determined. The data from each curve derived from triplicate independent assays are presented. Mean values are shown with error bars representing SEM. Data obtained were evaluated using professional statistic software GraphPad Prism 5.0.

Table 4

<table>
<thead>
<tr>
<th>Primer sequence</th>
<th>Recombinant plasmid</th>
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<tr>
<td></td>
<td>pET28a–hIDO2</td>
</tr>
<tr>
<td>F: 5'-AGAGGAGATATACATGCAGCCCGACAGACGAATGTGAAG-3'</td>
<td></td>
</tr>
<tr>
<td>R: 5'-GTGGTGGTGGTGCTCGAGCTAACCACGTGGGTGAAGATTGAC-3'</td>
<td></td>
</tr>
<tr>
<td>pcDNA3.1(+)–hIDO2</td>
<td></td>
</tr>
<tr>
<td>F: 5'-TTTAAACTTAAGCTTGGTACCGCCACCAGTTGCATTTTATATTAT-3'</td>
<td></td>
</tr>
<tr>
<td>R: 5'-TCTAGACTCGAGGCCGCCTAACCACGTGGGTGAAGATTGAC-3'</td>
<td></td>
</tr>
</tbody>
</table>

Endonuclease sites are emphasized with linear underline; Kozak sequence is emphasized with wavy underline. pET28a–hIDO2, digestion with Nco I and Xho I; pcDNA3.1(+)–hIDO2, digestion with Kpn I and Not I.
4.5. Enzymatic activity of purified hIDO2

Determination of the kinetic parameters $K_m$ and $k_{cat}$ was performed by incubating hIDO2 (2 μM) with two-fold serially diluted L-Trp (40,000 μM–0.25 μM) as substrate and the experiment was carried out using the same protocol reported by Pantouris et al. [30]. Kinetic parameters were determined by fitting to a non-linear form of the Michaelis–Menten equation using GraphPad Prism 5.0.

4.6. Enzymatic IDO2 inhibition assay

Based on the methods of Takikawa et al. [41] and Austin et al. [42], the tryptophan catabolizing activity and enzyme kinetic properties of IDO2 were characterized with mild alterations. Similar to IDO1, IDO2 is also prone to autoxidation. Herein, to maintain the enzyme activity, ascorbic acid (reductant), methylene blue (electron carrier) and catalase (to avoid inhibition of IDO2 by H$_2$O$_2$) were supplemented in the bioassay system. To screen IDO2 inhibitor candidates, each component was dissolved in 50 mM potassium phosphate buffer, pH 7.5. Each assay solution contained 500 μL of reaction mixture comprising 40 mM ascorbic acid, 200 μg/mL catalase, 20 μM methylene blue, substrate i-tryptophan (0–40 mM), the inhibitor candidate (10 μM for preliminary screening and appropriate concentration gradients for $K_i$ and IC$_{50}$ value determination) and the enzyme at a final concentration of 1500 nM. The assays were conducted at 37 °C for 30 min in thermostatically controlled water bath and terminated by addition of 200 μL of trichloroacetic acid (30% w/v). To promote the formation of i-kynurenine, an additional incubation at 65 °C for 15 min in water bath was carried out. Each reaction was conducted in triplicate. After centrifuging for 15 min at 13,000 × g, 4 °C, 100 μL of the supernatant from each tube was transferred into 96-well microplates and mixed with the equal volume of 4% (w/v) trichloroacetic acid (DMAB) in acetic acid (2% w/v). The assay products generated from the dioxygenation of L-Trp catalyzed by IDO2 were confirmed by measuring their absorbance at 492 nm at which wavelength the kynurenine-DMAB complex has an absorption peak. In the assays, the controls were included to eliminate interference, such as any reactions of the inhibitor molecules with the substrate or the substrate with DMAB in the absence of IDO2. For the screening of IDO2 inhibitors, the inhibitory activities of typical competitive inhibitors L-1-MT and D-1-MT were measured under the same conditions as controls.

4.7. Determination of the inhibition type and kinetic parameters

To analyze the data obtained from the above assays, kinetics constants $K_i$ were determined by plotting $[S]/V$ against inhibitor concentrations, where $[S]$ and $V$ represented the substrate concentration and the reaction rate, respectively. On the other hand, the type of inhibition was evaluated via plotting reaction velocity $V$ against enzyme amount [E]. The substrate (i-tryptophan) concentrations varied from 20 mM to 40 mM; while concentrations of inhibitors varied over a two-fold range above and below the concentration yielding approximately 50% inhibitory activity. Enzymatic IC$_{50}$ values of the inhibitors studied were measured using data analysis software GraphPad Prism 5.0.

4.8. Cellular IDO2 inhibition assay

To study the cellular hIDO2 inhibition of candidate compounds, recombinant plasmid pcDNA3.1(+) hIDO2 was constructed and transfected into human glioblastoma U87 MG cells which had no IDO1 expression (confirmed by RT-PCR and western blot) therefore eliminated the interference of IDO1. U87 MG cells were cultivated in DMEM containing 50 U/mL penicillin, 50 mg/mL streptomycin, 4500 mg/L glucose, and 10% inactivated FBS at 37 °C with 5% CO2 and 95% humidity. When a cell density of 80% confluent monolayer was reached, U87 MG cells were transfected with pcDNA3.1(+) hIDO2 using the transfection reagent Lipofectamine 2000 according to the manufacturer’s instructions. An empty pcDNA3.1(+) expression vector was served as control. After 18 h of incubation, the transfected cells were seeded in 96-well culture plates at a density of 2.5 × 10³ cells/well in a final volume of 200 μL supplemented with 200 μM L-Trp. A serial dilution of the tested compounds was added to the culture medium after an additional 6 h of incubation. The reaction was terminated by addition of 30% (w/v) trichloroacetic acid (10 μL for 140 μL of the reaction mixture) 24 h later. The plates were incubated at 65 °C in water bath for 15 min to facilitate the transformation of N-formylykynurenine to i-kynurenine, followed by centrifugation at 13,000 × g for 10 min to remove the sediments. 100 μL of the supernatant were then transferred to another 96-well plate and mixed with a same volume of 2% (w/v) 4-dimethylaminobenzaldehyde in acetic acid. The percentages of inhibition of tryptophan degradation or kynurenine production by the compounds were calculated by measuring the absorption at 492 nm using a microplate reader. Cellular IC$_{50}$ values were determined via non-linear regression analysis using GraphPad Prism 5.0.

4.9. PCR analysis of hIDO2 mRNA expression

The expression of hIDO1 and hIDO2 mRNA in the U87 MG cells transfected or not transfected by pcDNA3.1(+) pcDNA3.1(+) hIDO2 was evaluated by polymerase chain reaction (PCR). 24 h after the transfection, the total RNA was isolated from the cells using the Trizol reagent (Invitrogen, Japan). Next, reverse transcription was performed to obtain hIDO1 and hIDO2 cDNA which were then amplified by PCR using specific primers (hIDO1: forward: 5'-tgccaatccacagaaatg-3', reverse: 5'-gattgccaagacacagtctg-3'; hIDO2: using the primers described in Table 4). The PCR products were analyzed on a 1% agarose gel with GAPDH as an internal standard.

5. Western blot analysis

The U87 MG cells were harvested 24 h after the transfection and incubation, and the cells were lysed and analyzed by western blot using the same methods as previously described [38]. Anti-IDO2 (Catalog No. sc-87164) and anti-GAPDH (Catalog No. AG019) antibodies were purchased from Santa Cruz and Beyotime Biotechnology, respectively, and used according to manufacturers’ instructions.

Author contributions

J.L. performed most of the in vitro, cellular, and related experiments. Y.L., Z.G., D.Y. and N.H. helped with resolving experimental technical problems and data analyzing. C.K. synthesized tryptanthins and analyzed chemistry and structure-related results. Q.Y. and C.K. developed the experimental instructions. J.L. performed most of the experiments and analyzed data, and wrote the manuscript.

Disclosure

The authors have no conflicts of interest to declare.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2016.07.013.

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