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Heme-containing enzymes and inhibitors for tryptophan metabolism

Daojing Yan,[a] Ying-Wu Lin[b] and Xiangshi Tan*[a]

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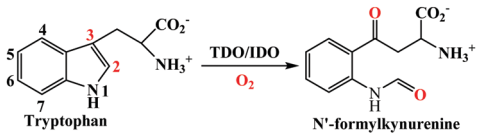
Iron-containing enzymes such as heme enzymes play crucial roles in biological systems. Three distinct heme-containing dioxygenase enzymes, tryptophan 2,3-dioxygenase (TDO), indoleamine 2,3-dioxygenase 1 (IDO1) and indoleamine 2,3-dioxygenase 2 (IDO2) catalyze the initial and rate-limiting step of L-tryptophan catabolism through the kynurenine pathway in mammals. Overexpression of these enzymes causes depletion of tryptophan and the accumulation of metabolic products, which contributes to tumor immune tolerance and immune dysregulation in a variety of disease pathologies. In the past few decades, IDO1 has garnered the most attention as a therapeutic target with great potential in cancer immunotherapy. Many potential inhibitors of IDO1 have been designed, synthesized and evaluated, among which indoximod (D-1-MT), INCB024360, GDC-0919 (formerly NLG-919), and an IDO1 peptide-based vaccine have advanced to the clinical trial stage. However, recently, the roles of TDO and IDO2 have been elucidated in immune suppression. In this review, the current drug discovery landscape for targeting TDO, IDO1 and IDO2 is highlighted, with particular attention to the recent use of drugs in clinical trials. Moreover, the crystal structures of these enzymes, in complex with inhibitors, and the mechanisms of Trp catabolism in the first step, are summarized to provide information for facilitating the discovery of new enzyme inhibitors.

1. Introduction

Iron, one of the essential elements for biological systems, usually forms complexes such as heme (iron protoporphyrin IX) and iron–sulfur clusters, and is used by many proteins to perform diverse functions, including electron-transfer, oxygen delivery, catalysis and signaling.[1] L-Tryptophan (Trp) is the least abundant of the essential amino acids, which accounts for only 1% of the total amino acid content,[2] with plasma concentrations of approximately 40–80 μM in humans.[3] It has three roles in the human body: general protein synthesis; synthesis of neurotransmitter-serotonin; and catabolism through the kynurenine pathway.[4] This pathway, as first described in 1947, is responsible for the metabolism of approximately 95% of all mammalian dietary tryptophan.[5,6]

Tryptophan 2,3-dioxygenase (hTDO) and indoleamine 2,3-dioxygenase (hIDO) are the only two kinds of heme-containing dioxygenases in humans.[1] TDO, IDO1 and the recently discovered IDO2, comprise a family of enzymes,[2] catalyzing the oxidation of L-Trp to *N*′-formylkynurenine (NFK), the first- and rate-limiting step in tryptophan catabolism. In mechanism, the enzyme incorporates O_2 and cleaves the C2–C3 bond of the indole ring of the substrate Trp (Scheme 1). Subsequently, NFK catabolism generates L-kynurenine (KYN), which is further converted to other important bioactive metabolites, including kynurenic acid, quinolinic acid, ATP and the coenzyme nicotinamide adenine dinucleotide (NAD), an essential sustaining redox cofactor in living systems.[4,7,8]

Generally, tryptophan metabolic enzymes benefit normal physiological metabolism, due to the metabolites of Trp.[9,10] However, overexpression of these enzymes results in the depletion of Trp in the local microenvironment and subsequent inhibition of T cell responses.[11] T cells sense low Trp levels *via* uncharged tRNAs, and subsequently activate kinase general control nonderepressible 2 (GCN2) and initiate an amino acid starvation response, leading to cycle arrest, anergy, and increased sensitivity to apoptosis.[8] Therefore, Trp metabolism along the kynurenine pathway is closely related to the regulation of immune responses. In addition, several studies have proposed that immunosuppression by Trp degradation is not solely a consequence of lowering local Trp levels, but also of accumulating high levels of Trp metabolites.[12]



Scheme 1 The dioxygenase reaction of Trp catalyzed by TDO or IDO, producing *N*′-formylkynurenine.

[a] *Department of Chemistry & Institutes of Biomedical Sciences, Fudan University, Shanghai 200433, China. E-mail: xstan@fudan.edu.cn*
[b] *School of Chemistry and Chemical Engineering, University of South China, Hengyang 421001, China*

Platten's group demonstrated that KYN is an endogenous ligand of the aryl hydrocarbon receptor (AHR), the receptor for the environmental toxin and known carcinogen, dioxin.[11] Thus, TDO/IDO, as the first- and rate-limiting step of Trp metabolism, is an attractive target for immunity and anticancer therapy. Hence, it has great significance in the investigation of efficient TDO/IDO inhibitors for the treatment of diseases.

2. Discovery of TDO and IDO1/2

TDO was discovered in 1936 and described as being both eukaryotic and prokaryotic.[13] It is highly specific for the substrate L-Trp and some derivatives substituted in the 5- and 6-positions of the indole ring.[14] This enzyme was originally identified in the liver and is expressed in many other cells and organs, including the placenta, pregnant uterus, maternal and embryonic tissues in early concepti, epididymis, testis and brain.[8] Its expression is up-regulated by dietary Trp levels, glucocorticoids and KYN.[15] The crystal structure of TDO[15–17] reveals that it is a tetramer with a molecular weight of ~190 kDa (eukaryotic) and ~120 kDa (prokaryotic).[18]

IDO1 is a monomeric enzyme that was first isolated from rabbit intestine in 1967 as a D-Trp-metabolizing enzyme[19] with a molecular weight of 45 kDa.[10] IDO1 exhibited a much broader substrate acceptance than TDO, including L-Trp, D-Trp, 5-hydroxy-Trp, tryptamine, and serotonin, and was therefore named indoleamine 2,3-dioxygenase.[17] Moreover, it has a much wider distribution than TDO, and has been found in most tissues except the liver, including the stomach, intestines, colon, kidney, spleen, lung, and brain.[4] Numerous cytokines, including interferons (IFNα, IFNβ, and IFNγ), interleukins (IL-1 and IL-2), and tumor necrosis factor (TNF), serve as signaling molecules to induce the expression of IDO1.[20]

In 2007, three research groups independently reported the discovery of an additional tryptophan metabolic enzyme, namely IDO2.[21–23] IDO2 is a paralogue of IDO1 and is also capable of catalyzing the conversion of L-Trp to *N*′-formylkynurenine. It is constitutively expressed within the liver, kidney tubules, spermatozoa, and antigen-presenting dendritic cells,[24] with a molecular weight of ~45 kDa.[25] Metz *et al.* found that IDO2 is necessary for the induction of several pivotal inflammatory cytokines, including granulocyte-macro-phage colony-stimulating factor (GM-CSF), G-CSF, IFN-γ, TNF-α, IL-6, and monocyte chemoattractant protein-1 (MCP-1/CCL2), which is unaffected by the loss of IDO1.[26] Meanwhile, common polymorphisms inactivate the functional activity of IDO2 by affecting approximately 50% of the Caucasian population and 25% of the African population.[22,27]

Although IDO2 has been identified as a Trp dioxygenase, it is much less effective in this role than IDO1, owing to its significantly lower substrate binding affinity and lower turnover rates.[28] Thus, the physiological role of IDO2 remains unclear.[6] Note that soluble human IDO2 is difficult to express with enzymatic activity; attempts to produce and purify it from both mammalian and bacterial expression systems have been made.[29,30] Mouse IDO2 shares 72% overall sequence identity with human IDO2, and 100% sequence identity in the active site. Therefore, mouse IDO2 was generally used for inhibitor selection.[30] Meanwhile, mouse IDO2 exhibits higher catabolic activity compared to human IDO2. Recently, Li and co-workers[25] successfully expressed and purified human IDO2 and established an IDO2 bioassay system to investigate its function.

Commonly, these three enzymes use heme as a prosthetic group, and require the reduction of heme for optimal enzymatic activity.[15,24,31] However, an alignment of TDO and IDO sequences is only possible based on their structures, which show a sequence identity as low as 10% between them.[16] Nevertheless, IDO1 and IDO2 share ~43% similarity at the amino acid level (for human and mouse proteins), and they are encoded by genes adjacent to each other, on chromosome 8 in humans and mice.[32]

3. Crystal structures of the IDO-complex and TDO-complex

To date, three crystal structures of the human IDO1-complex have been discovered.[31,33,34] In 2006, Sugimoto and co-workers[31] first reported the crystal structures of recombinant human IDO1 in complex with 4-phenylimidazole (PI) (PDB code 2D0T, Fig. 1A) and cyanide ion forms (PDB code 2D0U). The structure of IDO1 provided insight into the catalytic reaction and structural-based inhibitor design. The overall structure showed that IDO1 was folded into two distinct domains. In the PI-bound X-ray structure, the ligand is bound in a deep binding site with its phenyl ring inside a hydrophobic pocket (pocket A), and the imidazole group of PI coordinates to the heme iron directly. Another hydrophobic pocket (pocket B) at the entrance of the binding site is occupied by buffer molecules of *N*-cyclohexyl-2-aminoethanesulfonic acid (CHES) (Fig. 1A). The authors used site-directed mutagenesis to identify the important amino acid residues responsible for the catalytic reaction. They showed that mutation of the Phe226, Phe227 and Arg231 residues drastically reduced the dioxygenase activity, indicating that these residues are essential for substrate recognition by hydrophobic interactions.

Based on the crystal structure of the IDO1–PI complex where pocket A was occupied by PI and pocket B was fitted by two buffer molecules, Tojo and co-workers[34] considered that PI was a fragment-like inhibitor and pocket B could be targeted to improve the binding affinity for finding potent IDO1 inhibitors. They then used a thiazolotriazole compound, Amg-1,[29] and rationally designed a series of imidazothiazole derivatives as potent inhibitors. Fortunately, they resolved the crystal structures of human IDO1/Amg-1 (PDB code 4PK5) and IDO1/**13b** complexes (PDB code 4PK6). The results revealed that both Phe226 and Arg231 contribute to the increased potency of inhibitors to IDO1, which is in accordance with the results of previous site-direct mutagenesis analysis.[31] Furthermore, Tojo and co-workers[34] first established the structure–activity relationships for pocket B, and indicated that pocket B is also essential for the selection of inhibitors. The pK_a values further suggested that the strong basicity of the nitrogen atom would

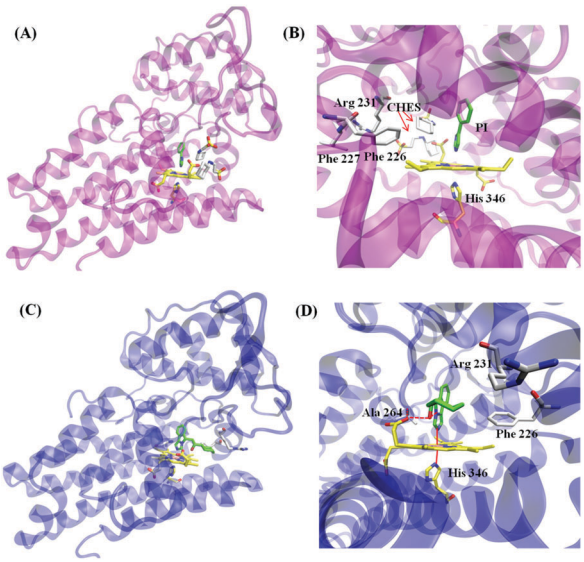


Fig. 1 Crystal structures of IDO1–inhibitor complexes. (A and C) Are the overall structure of human IDO1 (PDB: 2D0T and 5EK3), respectively;[31,33] (B) the active site of IDO1 with PI. Inhibitor PI is shown in green. The heme and a proximal His346 residue are shown in yellow. Two buffer molecules of CHES, Phe226, Phe227 and Arg231 are shown in white; (D) the active site of IDO1 with **24**.[33] The red dashed lines indicate the hydrogen bond network, concluding the 7-propionic acid of heme and the main chain NH group of Ala264 beside an intramolecular hydrogen bond within **24** by the isoindole nitrogen and the hydroxyl group. **24** is shown in green. The heme and proximal His346 residue are shown in yellow. Two essential residues, Arg 231 and Phe 226, in the active site are shown in white.

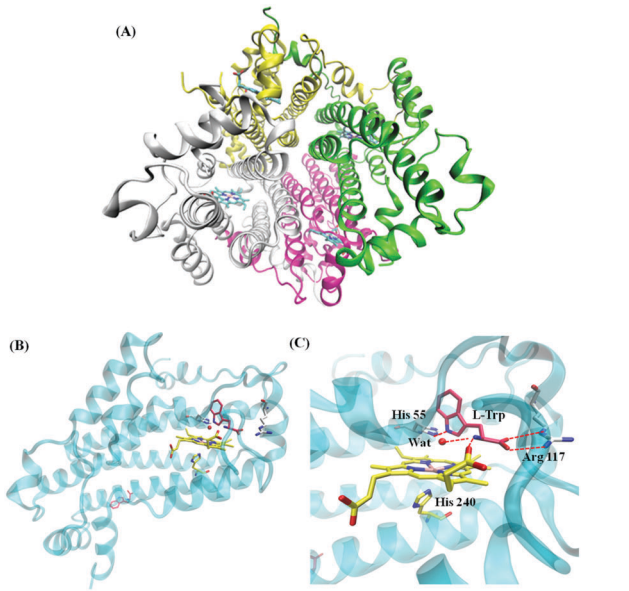


Fig. 2 The crystal structure of TDO. (A) Schematic representation of the tetramer RmTDO (PDB code 2NOX).[17] The four monomers are shown in green (chain A), yellow (chain B), white (chain C), and magenta 2 (chain D), respectively. Four hemes are shown in cyan; (B) schematic representation of the monomer XcTDO (PDB code 2NW8).[16] Two L-Trp are shown in red. One of them is located in the distal pocket of the protein at position 307. Another is located in the interface of the protein at position 308; (C) the active site of XcTDO with L-Trp. A proximal His240 residue and heme are shown in yellow. The residues mentioned in this article, His55 and Arg117, are shown in white. Arg117, L-Trp and heme form a hydrogen bond network shown by red dashed lines. A water molecule is located in the active site and is shown in red.

result in strong binding to the heme iron of IDO1, indicating that these compounds have potent IDO1 inhibitory activity.

As mentioned above, the reported IDO1 structures revealed that the enzyme binds the inhibitor with a comparatively weak affinity (in about the micromolar range). Therefore, the structures of IDO1 in complex with potent inhibitors need to be discovered. Excitingly, Peng[33] recently reported several IDO1-complex structures, and the inhibitory potency of the inhibitors was in the nanomolar range (PDB codes 5EK2, 5EK3 (Fig. 1B), 5EK4, and 5ETW). A distinct hydrogen bond network was discovered in the structure of IDO1 in complex with **24**, a NLG919 analogue containing an imidazoleisoindole core. The structure showed that the 7-propionic acid group of the heme and the main chain NH group of Ala264 form two intermolecular hydrogen bonds with **24**, which was not observed in other IDO1-complex structures. Structural biology studies of **24** and its analogues revealed that the weaker coordination covalent interactions and the loss of the hydrogen-bond network completely eliminate the capacity of these analogues to inhibit IDO1. Consequently, these investigations showed that the intramolecular hydrogen bond plays a unique role in drug discovery. It should be noted that although these structures provided information for the active site residues and the coordination state of inhibitors, they did not provide information regarding the substrate L-Trp, which is a missing piece in our understanding of the structure-based drug design of new IDO inhibitors.

Fortunately, TDO, another enzyme, catalyzes the initial step in Trp catabolism, and its structure in complex with L-trptophan has been reported.[15–17] Forouhar *et al.*[16] reported the crystal structures of reduced (Fe(II)) TDO from *Xanthomonas campestris* in a binary complex with the substrate L-Trp or 6-fluoro-Trp. The structure of XcTDO was first determined (PDB codes 1YW0 and 2NW8 (Fig. 2B)). In addition, they determined the structure of IDO from *Shewanella oneidensis* in the absence of the heme group (PDB code 1ZEE). Human IDO and *S. oneidensis* IDO were structurally homologous, although the sequence identity was only ~14%,[17] which makes reference for Trp binding to IDO. In the same year, Zhang *et al.*[17] resolved the crystal structure of TDO from *Ralstonia metallidurans* with the heme bound in the active site at 2.4 Å. (Fig. 2A). The structure is a tetramer with a heme bound at each active site. The monomeric fold and the heme binding site are similar to those of the large domain of IDO1. Excitingly, the first crystal structure of a eukaryotic TDO from *Drosophila melanogaster* in complex with heme was reported in 2013 by Huang *et al.*[15] (PDB code 4HKA). Compared with the prokaryotic TDOs, *Dm*TDO adopts a similar tetrameric architecture, but contains a unique small domain that interacts with the active site of an adjacent monomer and plays a role in catalysis. The *Xc*TDO mutants' crystal structures in a binary complex with L-Trp have also been reported,[35] which revealed that His55 is not essential for turnover, but greatly disfavors the mechanistically unproductive binding of L-Trp to the oxidized enzyme and allows the control of catalysis.

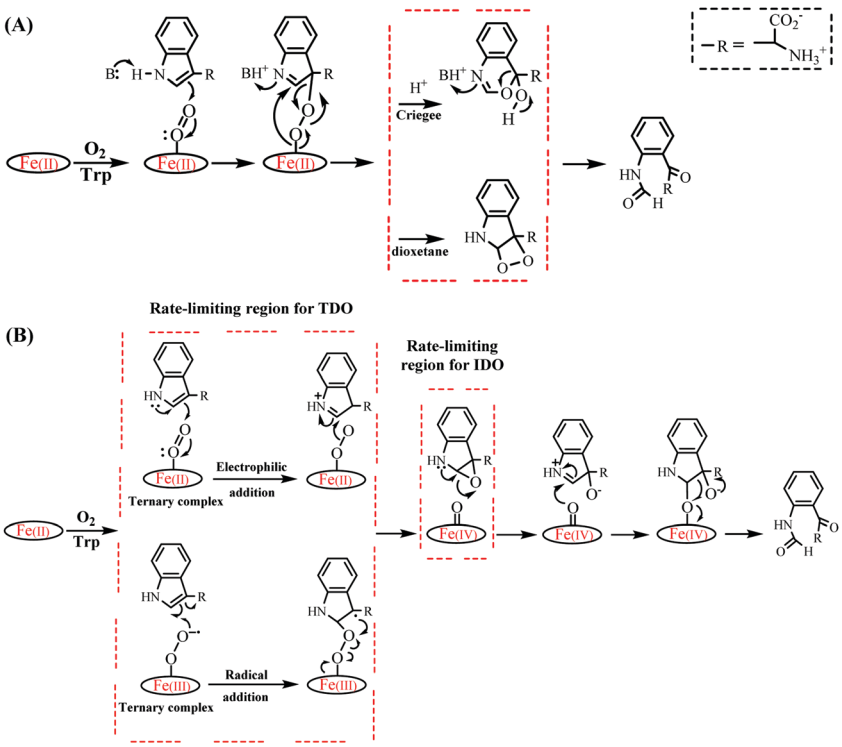
As mentioned above, TDO and IDO have a low sequence identity (10%).[16] The biochemical studies had shown that prokaryotic and eukaryotic TDOs exhibit notable differences in their biochemical properties. *Xc*TDO shows activity towards only L-Trp, and its binding affinity for L-Trp is 145-fold higher than that for D-Trp,[16] whereas *Hs*TDO shows a high activity for L-Trp, with a low activity for D-Trp.[36,37] These observations suggest differences in substrate recognition and catalysis between prokaryotic and eukaryotic TDOs. However, the structure of prokaryotic or eukaryotic TDO–Trp complex can still make reference for investigating the structure-based drug design with the aim of discovering potent inhibitors of IDO1. Note that a new discovery of IDO1 homogenous protein, IDO2, was first disclosed by Metz *et al.* in 2007.[22] To our knowledge, the structure of IDO2 has not been reported so far, and this should be the focus of future scientific research.

4. Progress in understanding Trp oxidation

Understanding the mechanism of Trp oxidation is essential from a clinical perspective, due to the key process in Trp catabolism.[38] Despite decades of investigation, the reaction mechanism of the dioxygenases is still not fully clarified. Specifically, the catalytic mechanism for Trp has a long history. In 1957, Hayaishi[39] demonstrated that O_2-dependent oxidation of Trp led to the formation of NFK, and two oxygen atoms from O_2 were incorporated into the substrate during this process. Early studies[40] suggested that the mechanism involved base-catalyzed deprotonation of the indole NH group as the initiating step. The reaction proceeds to yield the product by either a Criegee-type rearrangement or through the dioxetane intermediate (Scheme 2A).[41] However, Chung's group[42] performed density functional theory (DFT) calculations of a heme-Trp model system, which seriously challenged the base-catalyzed mechanism. The DFT study illustrated the energetic feasibility of forming a ferryl-type intermediate, which was supported by a Lewis-Ballester group using continuous-flow resonance Raman (RR) spectroscopy.[1] They demonstrated that the two atoms of O_2 are inserted into the substrate one at a time *via* a two-step reaction. Moreover, the ferryl-based mechanism was proposed and supported by other groups.[38,43] The mechanisms for dioxygenase catalysis have been well-reviewed in the literature.[44,45] Generally, it has been widely presumed that IDO and TDO oxidize substrates using the same mechanism, even though there is no evidence to support this assumption. However, Basran and coworkers concluded that the rate-limiting step in the TDO mechanism was different from that in IDO (Scheme 2B).[46] They found that a ternary [Fe(II)–O_2, L-Trp] complex accumulated during turnovers instead of ferryl-type intermediate accumulation, which complemented the ferryl-based mechanism of Trp metabolism.

5. Progress in the design of IDO/TDO inhibitors

The discovery of the crystal structures of the IDO-complex and TDO-complex facilitated the design of new enzyme inhibitors,[15–17,31]



Scheme 2 The mechanism of tryptophan oxidation. (A) A base-catalyzed abstraction mechanism followed by either Criegee or dioxetane rearrangements. (B) The difference of TDO and IDO in the rate limiting region.

based on the structure-based drug design (SBDD).[33,34] Subsequently, a molecular docking technology based on structures was used to understand the binding mode between the enzyme and the inhibitor.[47,48] In addition, high-throughput screening (HTS) was shown to be efficient in the selection of enzyme inhibitors.[49,50] Furthermore, a new structure-based virtual screening strategy was used for inhibitor selection.[51] It is worth noting that among a number of the inhibitors identified, many compounds are Trp analogues or heme binders like PI.[52] With new methods, new inhibitor scaffolds have been discovered by natural product screening, by SBDD or, in the majority of cases, by HTS over the past 10 years.[52] These methods accelerate the progress in discovery of new inhibitors. In this section, we will primarily focus on recently investigated potential inhibitors of TDO, IDO1 and IDO2, with the structures shown in Tables 1–3.

5.1 TDO inhibitors

TDO is the earliest discovered enzyme with the function of catalyzing L-Trp oxidation.[13] Nevertheless, only a few classes of TDO inhibitors have been reported to date. Some of them were identified using HTS technology or synthetic modifications based on the structure of the substrate.[53–55] A new strategy, structure-based virtual screening approach was used to identify novel TDO inhibitors.[51] The structures of these inhibitors and their IC_{50}/K_i values are summarized in Table 1. The K_i value is the inhibition constant of a substance, and the IC_{50} value expresses the concentration of inhibitor required to produce 50% inhibition of an enzymic reaction at a specific substrate concentration. These values have been determined to study the effect of drug concentration on the rate of reaction of an isolated enzyme.[56]

Two typical inhibitors are discussed here. In Pantouris' work,[55] a natural flavonoid compound **2** was targeted by HTS. Although **NSC 26326** (K_i = 0.03–0.07 μM) has a ~500 fold lower inhibition constant than **2** (K_i = 16.3 μM) *in vitro*, **2** had higher selection to TDO. Another potential inhibitor **6** with IC_{50} in a nanomolar range (30 nM) was selected by virtual screening and structural modification.[51] In this study, the selection of enzyme and cancer cell lines was tested, which showed promise for further investigation into TDO-related targeted therapies including cancer therapy.

5.2 IDO1 inhibitors

Since IDO1 has been described as a potent target in cancer treatment, the investigation for IDO1 inhibitors is ongoing intensively in academia and in pharmaceutical companies. Several potent IDO1 inhibitors with a structural diverse were reviewed before 2015.[27,52,59] The potent inhibitors reported recently are summarized in Table 2, which provides a reference for studying the chemical structure and inhibition activity of

Table 1 Inhibitors for TDO

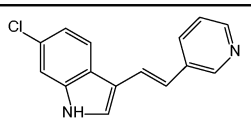
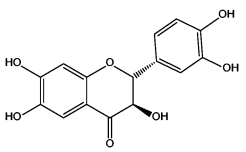
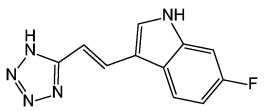
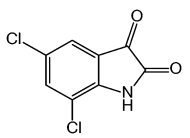
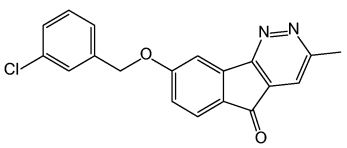
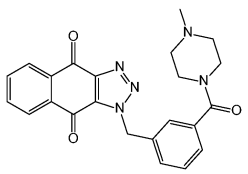
No.	Compound name	Compound structure	Value of IC_{50}/K_i (μM)	Ref.
1	**2** Chloro-3-(2-pyridin-3-ylvinyl)-1*H*-indole		IC_{50} = 5	54
2	**NSC 36398** Dihydroquercetin, taxifolin		K_i = 16.3	55
3	**58** 3-(2-(Pyridyl)ethenyl)indole		IC_{50} = 2	53
4	**8** Isatin derivative		K_i = 0.8	57
5	**9a** 3,8-Substituted 5*H*-indeno[1,2-*c*]pyridazin-5-one derivative		K_i = 0.11	58
6	**11e** Naphthotriazoledione derivative		IC_{50} = 0.03	51

Table 2 Inhibitors for IDO1

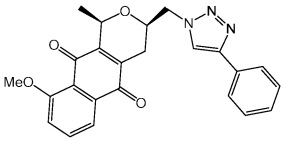
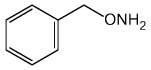
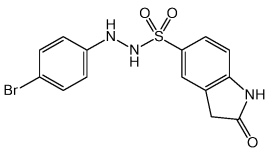
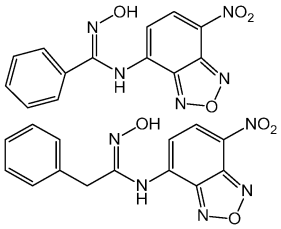
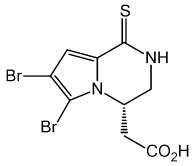
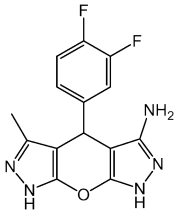
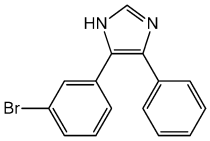
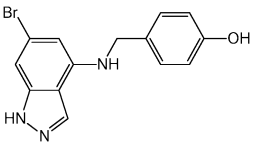
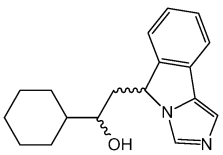
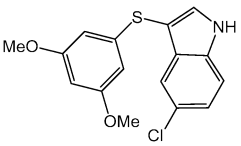
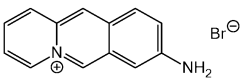
No.	Compound name	Compound structure	Value of IC_{50} (μM)	Ref.
1	**8** Pyranonaphthoquinone		IC_{50} = 6.0	64
2	*O*-Benzylhydroxylamine		IC_{50} = 0.9	65
3	**40** Phenyl benzenesulfonylhydrazides		IC_{50} = 0.036	62
4	*N*′-Hydroxybenzimidamides **1** *N*′-Hydroxy-2-phenylacetimidamides **2**		IC_{50} = 0.268 IC_{50} = 0.307	66
5	**22** Pyrrolopiperazinone alkaloid		IC_{50} = 92.1	67
6	**4l** Fused heterocyclic compound		IC_{50} = 0.151	68
7	**8** 4,5-Disubstituted imidazole		IC_{50} = 1.5	69
8	**2g** 1*H*-Indazole derivatives		IC_{50} = 5.3	47
9	**24** GDC-0919 analogue		IC_{50} = 0.038	33
10	**21** Arylthioindole		IC_{50} = 7	70
11	**2c** Natural compound		IC_{50} = 0.164	71

Table 2 (*continued*)

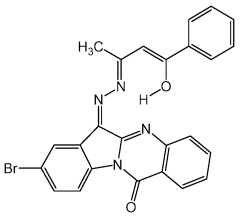
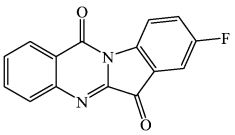
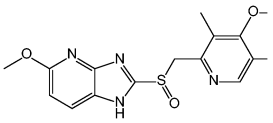
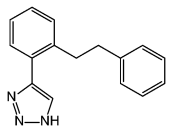
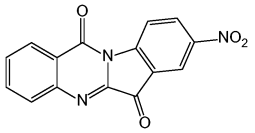
No.	Compound name	Compound structure	Value of IC_{50} (μM)	Ref.
12	**3c** Tryptanthrin analogue		IC_{50} = 7.14 (MCF-7 cell)	72
13	**Galanal**Natural compound	Not mentioned	IC_{50} = 7.7	73
14	**5c** Tryptanthrin derivative		K_i = 0.161	63

Table 3 Inhibitors for IDO2

No.	Compound name	Compound structure	Value of IC_{50} (μM)	Ref.
1	Tenatoprazole		IC_{50} = 1.8 (mouse IDO2)	77
2	**13a** 1,2,3-Triazole derivative		IC_{50} = 51 (mouse IDO2)	30
3	**5i** Tryptanthrin derivative		IC_{50} = 1.8 (human IDO2)	25

IDO1 inhibitors. Some of them are central pharmacophores that inhibitors primarily contain. Novel inhibitors containing carboranes have also been reported.[60,61]

As listed in Table 2, the IC_{50} values for the IDO1 inhibitors are below ten micromolar and some of them are even below tens of nanomolar. To date, **3** and **9** are the inhibitors with the highest potential reported for IDO1. They were all discovered by the National Health Research Institutes in Taiwan, and the potent *in vivo* pharmacodynamic activity and antitumor efficacy of **3** were proved.[62] This demonstrated 59% oral bioavailability and a 73% tumor growth delay without apparent body weight loss in the murine CT26 syngeneic model. Accordingly, **3**, was proposed as a potential drug lead worthy of advanced preclinical evaluation. GDC-0919 was developed by NewLink Genetics as an IDO1 inhibitor under clinical trial; meanwhile, its chemical structure has not yet been disclosed. Fortunately, an analogue of GDC-0919, **9**, was shown to be a potent inhibitor to IDO1 with an IC_{50} of 38 nM.[33] The IDO1-inhibitor crystal structure was first resolved with inhibition potency, and other imidazoleisoindole derivative cocrystal structures were also included. These structures elucidated the inhibition mechanisms of a series of compounds to IDO1, which were expected to facilitate the structure-based drug design of new IDO inhibitors. It should be noted that two natural compounds, **11** and **13**, have a value of IC_{50} as low as 7 μM, which suggests that natural products are also important resources for discovering potential IDO1 inhibitors. A natural compound, **14**, with high inhibition was reported by Yang's group.[63] The value of IC_{50} for the cells was determined to be 23 nM. Remarkably, it has therapeutic activity in Lewis Lung Cancer (LLC) tumor-bearing mice, which may be a potent candidate for entering clinical trials.

5.3 IDO2 inhibitors

Literature resources for IDO2 inhibitors are limited due to the discovery of this protein only ten years ago, or due to the fact that soluble human IDO2 is hard to express with enzymatic activity.[30] To our knowledge, there are only three articles referring to the structures of the inhibitor (Table 3). A tryptanthrin derivative, **3** (IC_{50} = 1.8 μM), is a novel human IDO2 inhibitor and can be used for potential therapeutic applications.[25] The physiological function of IDO2 was intensively investigated in biology using animal models, cells experiments and other methods.[2,74–76] The identification of a selective inhibitor of IDO2 for use as a tool compound could

be very useful in ongoing studies to elucidate the roles of IDO1 and IDO2 in inflammation and tumorigenesis.[6]

6. IDO1 inhibitors in clinical trials

Among the three enzymes involved in the first step of the kynurenine pathway, IDO1 has been the most extensively evaluated as an immunotherapy for cancer. However, recent studies have also begun to shed light on the potential role of TDO. Currently, IDO1, one of the Trp metabolic enzymes, stimulates academic research and pharmaceuticals companies with a wide range of interests to select its inhibitors for cancer immunotherapy. Hence, only IDO1 inhibitors in clinic will be discussed here.

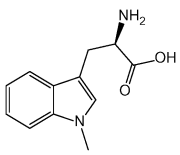
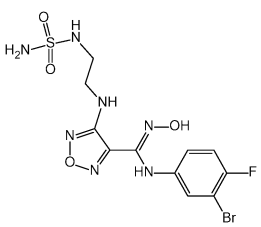
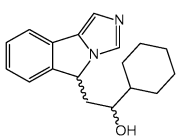
There is no IDO1 inhibitor approved by the US Food and Drug Administration (FDA) yet. Meanwhile, a few inhibitors for IDO1 are in the clinical trials stage (Table 4). Four agents, Indoximod (D-1-methyl-tryptophan, D-1-MT), INCB024360, GDC-0919, and an IDO1 peptide-based vaccine, are evaluated for their efficacy against a wide range of cancers, including metastatic diseases and solid tumors *etc.*[9] The results of the recent phase I–II studies suggest that D-1-MT, INCB024360 and/or IDO1-targeting vaccines are well tolerated by cancer patients, with clinical anticancer effects in a subset of patients.[78–80] 1-MT was first reported as an IDO inhibitor in 1991.[81] At the level of biochemical specificity, D-1-MT exhibited little biochemical activity as an IDO inhibitor relative to L-isomer. However, in preclinical efficacy studies, D-1-MT had superior antitumor activity compared with the L-isomer in preclinical models.[82] Eventually, D-1-MT was selected as a clinical candidate entering the clinical trials. The distinction between the two isomers is complicated, with the D-isomer exhibiting little biochemical activity as an IDO inhibitor relative to L-isomer. Interestingly, this phenomenon led to the discovery of IDO2, a paralogue of IDO1.[22] The Phase II studies of D-1-MT are recruiting.[9]

INCB024360, as developed by the Incyte Corporation through the HTS strategy, is a potent competitive inhibitor of IDO1 (HeLa IC_{50} = 19 nM).[49] The structure of INCB024360 was recently disclosed and is now commercially available.[52] Clinical studies have shown that INCB024360 is well tolerated, normalizes KYN levels effectively, and produces maximal inhibition of IDO1 activity at doses of >100 mg BID (twice daily). In preclinical models, single agent IDO1 inhibition was found to only slow tumor outgrowth.[80] Therefore, studies on INCB024360 in combination with chemotherapy or additional immune checkpoint inhibitors are ongoing (*i.e.* Warfarin[83] and pembrolizumab[84]).

Another clinical trial[85] reported that the *in vivo* IC_{50} of INCB024360 to inhibit IDO1 was estimated to be ~70 nM at a pharmacokinetics steady state, which agrees well with the experimentally determined *ex vivo* IC_{50} value. It was demonstrated that bioconversion of Trp to KYN is catalyzed by IDO1 and TDO in parallel, which complicated the determination of INCB024360's IC_{50} to inhibit IDO1. Then, external biological stimulants, lipopolysaccharides (LPS), and IFN-γ were introduced in experiments to selectively induce IDO1 expression and minimize the relative contribution of TDO. The model suggested that ~60% and ~40% of Trp to KYN bioconversion was mediated by IDO1 and TDO, respectively, demonstrating the essential role of combination with IDO and TDO inhibitors. It is worth mentioning that the KYN/Trp ratio was found to be a relevant blood-based biomarker for monitoring the inhibition of IDO1 *in vivo*.[80]

An imidazoleisoindole derivative, GDC-0919, developed by NewLink Genetics, is undergoing phase I clinical trials in the treatment of recurrent advanced solid tumors.[9,86] Its chemical structure is undisclosed; however, a similar structure is used as a reference.[33] An *in vivo* study revealed that oral administration

Table 4 Structures of IDO1 inhibitors in clinical trials

Compound name	Compound structure	Value of IC_{50} (μM)	Ref.
D-1-MT indoximod, NLG8189		NM	82
INCB024360 epacadostat		IC_{50} = 71 nM	91
GDC-0919 analogue		IC_{50} = 38 nM	25 and 33

NM = not mentioned. No IC_{50} value reported since D-1-MT exhibited little biochemical activity as an IDO inhibitor relative to L-1-MT.

of GDC-0919 reduces the concentration of KYN in plasma and tissue by ~50%.

The idea of employing vaccines as anticancer interventions was first theorized in 1890s by Paul Ehrlich and William Coley.[87] IDO1 peptide-based vaccine is a method of immunotherapy for cancer. The goal of immunotherapy is to marshal the specificity and long-term memory of the adaptive immune response to achieve durable tumor regression and a possible cure.[88] Vacchelli reviewed the clinical trial for peptide vaccines in cancer therapy, and mentioned that clinical trials testing IDO (NCT01219348) for Non-Small Cell Lung Carcinoma (NSCLC) and IDO surviving combined with GM-CSF, imiquimod and temozolomide (NCT01543464) for melanoma were recruiting.[87] In 2014, Iversen's group[89] reported the first in-human clinical study (NCT01219348). They demonstrated the clinical relevance of targeting IDO by a peptide vaccine strategy in 15 metastatic patients with stage III/IV NSCLC. Only in one patient was a partial response induced and another six patients showed long-lasting disease stabilization. The median OS was 25.9 months without severe toxicities observed. Based on these observations, they pointed out that therapeutic targeting of IDO may be an important vaccine strategy in metastatic NSCLC. Aris[90] represented the phase I clinical trial for malignant melanoma with metastasis treated by vaccine-peptide derived from the protein IDO (IDO Long), in combination with ipilimumab or vemurafenib (NCT02077114). The ongoing and historical clinical trials that target Trp catabolism in cancer were well summarized by Zhai and co-workers.[9] They mentioned that the IDO peptide-based vaccine was combined with other drugs for melanoma under recruiting.[9]

Recently, more and more clinical trial reports have suggested that combining Trp enzyme targeting with chemotherapy, radiotherapy and/or immunotherapy, may be an effective tool against a wide range of malignancies. They need further investigation gradually. It is worth mentioning that Van den Eynde and co-authors showed that pharmacological inhibition of both TDO and IDO1 to improve the efficacy of cancer immunotherapy would provide a complementary, rather than redundant, approach.[12]

7. Conclusions

As reviewed herein, TDO, IDO1 and IDO2 are crucial dioxygenases containing heme iron in biology, which are closely related to human health and disease. Numerous medicinal chemistry studies are currently aimed at the design of novel, potent, and selective inhibitors for these enzymes. Meanwhile, the emerging opportunities and the significant challenges associated with pharmacological modulation of these enzymes still need to be explored deeply. Two issues remain unresolved: (i) TDO/IDO alone as a target for treatment is not efficient due to the fact that they are all the key rate-limiting enzymes of Trp catabolism; and (ii) despite considerable advances in our understanding of this metabolic pathway, the molecular targets and mechanisms responsible for the biological effects of both Trp depletion and the accumulation of Trp metabolites are not clear yet.

Therefore, the combination of Trp enzyme targeting with chemotherapy, radiotherapy and/or immunotherapy, may be a viable approach to improving the efficiency of standard chemotherapeutic treatments. The combination of vaccines and drugs for treatment, and the differences in antibody specificity should be also considered. Furthermore, special attention should be directed to TDO/IDO dual-acting inhibitors as a new route for pharmacological research, as well as the toxic side effects of drugs.

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