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REVIEW ARTICLE

Highlights at the gate of tryptophan catabolism: a review on the mechanisms of activation and regulation of indoleamine 2,3-dioxygenase (IDO), a novel target in cancer disease

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Abstract Indoleamine 2,3-dioxygenase (IDO) catalyzes the first and rate-limiting step of Kynurenine pathway along the major route of Tryptophan catabolism. The scientific interest in the enzyme has been growing since the observations of the involvement of IDO in the mechanisms of immune tolerance and in the mechanisms of tumor immuno-editing process. In view of this latter observation, in particular, preclinical studies of small molecule inhibitors of the enzyme have indicated the feasibility to thwart the immuno-editing process and to enhance the efficacy of current chemotherapeutic agents, supporting the notion that IDO is a novel target in cancer disease.

This review covers the structural and conformational aspects of substrate recognition by IDO, including the catalytic mechanism and the so-far puzzling mechanisms of enzyme activation. Furthermore, we discuss the recent advances of medicinal chemistry in the field of IDO inhibitors.

Keywords Indoleamine 2,3-dioxygenase · Immune tolerance · Cancer · Kynurenine pathway · Tryptophan metabolism

Introduction

The amino acid L-Tryptophan (L-Trp, **1,** Fig. 1) is an essential metabolite in mammals and the least abundant of all essential amino acids. Its catabolism is accurately controlled by a number of metabolic pathways that lead to the formation of several biologically active compounds. These comprise the aminergic neurotransmitter serotonin (5-hydroxytryptamine, 5-HT, (**2**), the neurohormone melatonin (**3**), several neuroactive metabolites of melatonin, products of the kynurenine pathway (Fig. 2), and the amine tryptamine (**4**) (Ruddick et al. 2006). While the protein-related pool of L-Trp is kept constantly balanced between the processes of protein catabolism and protein synthesis, about the 99% of dietary intake of L-Trp is routed to the kynurenine pathway of tryptophan catabolism (Fig. 2) (Peters 1991).

This pathway is composed of two branches where the central metabolite L-Kynurenine (L-Kyn, **6**) is transformed, respectively, into the neurotoxic metabolite Quinolinic acid (QUIN, **10**), or into the neuroprotective metabolite Kynurenic acid (KYNA, **7**) (Stone and Darlington 2002). At the gate of kynurenine pathway of tryptophan catabolism one of the two enzymes, tryptophan 2,3-dioxygenase (TDO) and indoleamine 2,3-dioxygenase (IDO), catalyzes the initial and rate-limiting step of the pathway consisting in the oxidative cleavage of the pyrrole ring of the indole nucleus of L-Trp to yield *N*-Formylkynurenine (**5**) (Higuchi et al. 1963; Hayaishi 1993).

Although both enzymes are heme-containing proteins and catalyze the same reaction, TDO and IDO show a number of differences. TDO is mainly expressed in the liver and is induced by tryptophan, tyrosine, histidine, glucocorticoids and kynurenine (Taylor and Feng 1991). Conversely, IDO is ubiquitously expressed in many tissues and is regulated by complex immunological signals including type II interferons (IFN-γ), inflammatory cytokines, lipopolysaccharde (LPS) and tumor necrosis factor (TNF) (Grohmann et al. 2003; Mellor and Munn 2004). Whereas TDO is active as homotetramer and has a

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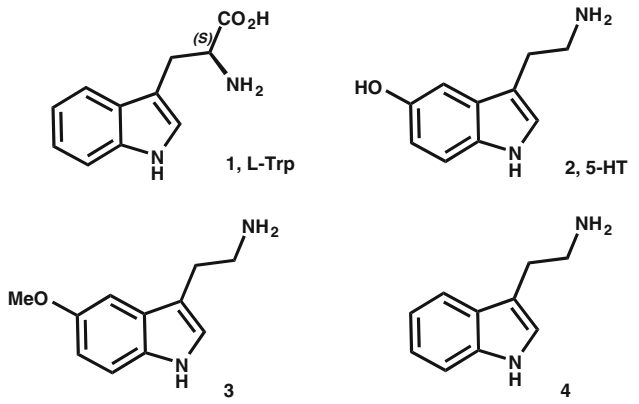


Fig. 1 Some biologically active end-products of L-Trp (**1**) metabolism

restricted substrate specificity to L-Trp, IDO functions as monomeric enzyme and binds other indole-bearing compounds, such as serotonin (**2**) and tryptamine (**4**), additionally to L-Trp (Sono et al. 1980, 1996). Interestingly, a novel enzymatic isoform of IDO has been recently described and termed indoleamine 2,3-dioxygenase-2 (IDO-2) (Ball et al. 2007, 2008). IDO and IDO-2 share a statistically significant sequence homology (43% identity) and are not structurally related to TDO. IDO-2 is predominantly expressed in murine kidney, liver, male and female reproductive system, where alike IDO, the enzyme catalyzes the oxidative cleavage of a broad range of indole-bearing substrates.

Since the discovery of the involvement of IDO in maintaining maternal tolerance toward the fetus (Munn et al. 1998), the scientific interest in the enzyme has been directed on its role in the generation of immune tolerance to foreign antigens. This area of investigation is reviewed in depth elsewhere (Fallarino et al. 2003; Grohmann et al. 2003; Puccetti and Grohmann 2007) and has led to associate the early observations of elevated expression of IDO in various human cancers to the participation of the enzyme in the tumor immuno-editing process which sets up peripheral tolerance to tumor antigens (Uyttenhove et al. 2003). In this framework, IDO promotes tumor outgrowth by counter-regulating inflammatory immune responses that would hamper tumor cell survival (Muller and Prendergast 2007; Katz et al. 2008). Results from preclinical studies of small molecule inhibitors of IDO have shown the feasibility to thwart the enzyme mediated immuno-editing process and to enhance the efficacy of current chemotherapeutic agents (Muller et al. 2005a, b). These data support the notion that IDO is a novel therapeutic target for the development of new cancer drugs (Muller and Scherle 2006; Muller and Prendergast 2007).

Very recently, an impairment of IDO activity following the lack of reactive oxygen species has also been associated to hyperinflammatory phenotypes that feature chronic granulomatous disease (Romani et al. 2008).

In light of the above observations, there is intense interest shown in understanding the mechanisms of activation and regulation of IDO, and in using such information to develop pharmaceutically suitable synthetic inhibitors that might be used in cancer therapy. Herein, we discuss the structural and conformational aspects of substrate recognition by IDO, highlighting the catalytic mechanism and the proposed mechanisms of enzyme activation. In addition, we report a concise overview of the different classes of inhibitors so far available as chemical tools to dissect the functions of the enzyme.

Structural and conformational aspects of substrate recognition

Crystallization studies of IDO have allowed unveiling the presence of two folding domains in the enzyme (Fig. 3) (Sugimoto et al. 2006). While the smaller domain contains nine α-helices and two β-sheets, the larger one is composed by 15 helices and contains the catalytic pocket. Four long helices and three short helices constitute the binding site of the heme cofactor with the third long helix providing the residue His346 at the fifth coordination site of the iron-heme. A broad number of interactions including salt bridges, hydrogen bonding and hydrophobic interactions spans the interface (3,100 A^2) between the two domains and stabilizes the structure of the enzyme above the sixth coordination site of the iron-heme. The access to the catalytic site is bordered by a flexible loop (360–380, not solved in the crystal structure) that defines a channel running parallel to the heme plane. Coarse graining simulations have indicated a role for such loop in controlling the shuttling of substrate and products to the catalytic site of IDO (Fig. 4). While the unbound state of the enzyme favors an open conformation of the loop, the substrate bound complex of IDO promotes the closure of the region 355–385, which further stabilizes the binding pose of the substrate within the catalytic site (Macchiarulo et al. 2007).

The sixth coordination site of the iron-heme constitutes the distal binding site where the substrate binds. This is composed of residues belonging to both the large and small domains and to a flexible loop (residues 260–265) connecting the two domains. Interestingly, the catalytic site of IDO is almost devoid of polar residues, with Ser167 being the only exception. Spectroscopic and kinetic data for mutant Ser167Ala, however, indicate that this residue is involved neither in substrate recognition, nor in catalytic activity (Chauhan et al. 2008).

Docking experiments of L-Trp (**1**) into the crystal structure of the ferric form of IDO, have evidenced that the

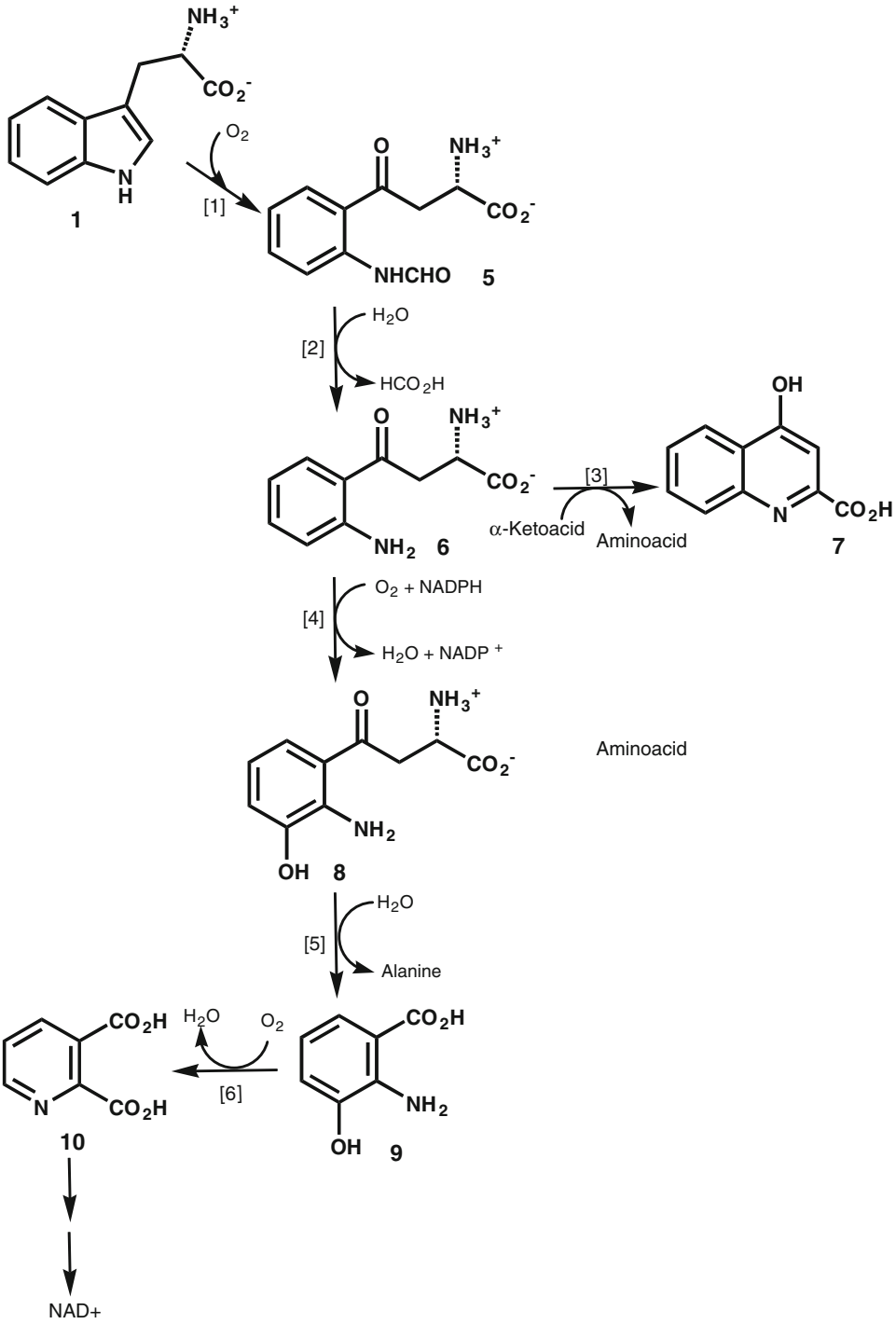


Fig. 2 The kynurenine pathway of tryptophan catabolism. **a** Compounds: L-Tryptophan (**1**), *N*-Formylkynurenine (**5**), L-Kynurenine (**6**), Kynurenic acid (**7**), 3-Hydroxykynurenine (**8**), 3-Hydroxyanthranilic acid (**9**), Quinolinic acid (**10**). **b** Enzymes: Tryptophan or Indolemanine 2,3-dioxygenase [1], Formamidase [2], Kynurenine Amino-transferase [3], Kynurenine-3-monooxygenase [4], Kynureninase [5], 3-Hydroxyanthranilate-3,4-dioxygenase [6]

substrate correctly binds the enzyme if a superoxide or oxygen moiety is already docked into the sixth coordination site of the iron-heme (Macchiarulo et al. 2007). According to the proposed binding mode (Fig. 5), the indolic ring of the substrate is packed with a flexible loop (residues 260–265) through hydrophobic interactions. This highly conserved loop has been indicated as a key element in affecting the conformational equilibrium between the active and inactive forms of the enzyme (Papadopoulou et al. 2005; Samelson-Jones and Yeh 2006). The amino-acidic moiety of L-Trp faces the entrance of the enzyme where the alpha amino group interacts with the 7-propionate moiety of the heme, and the alpha carboxylic group points toward Arg231 that, upon a conformational rearrangement, may promote the formation of a salt bridge interaction. Noteworthy, mutagenesis experiments have

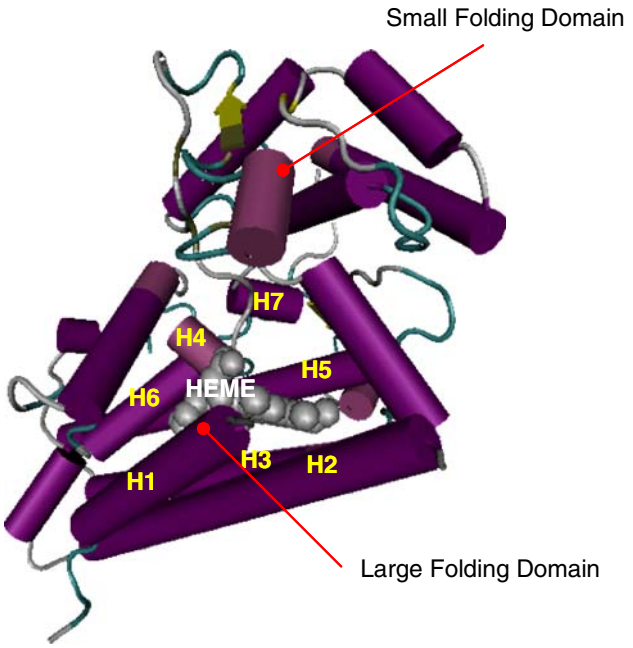


Fig. 3 Crystal structure of the inactive form of IDO (pdb code: 2D0T). The two folding domains of the enzyme are labeled

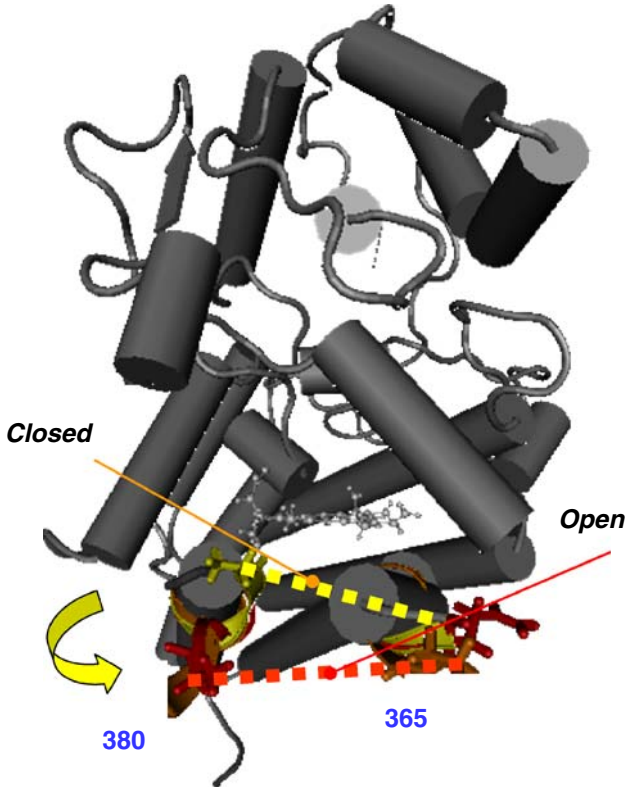


Fig. 4 A flexible loop (residues 360-380, not solved in the crystal structure) borders the access to the catalytic site of IDO. An open (*yellow*) and closed (*red*) conformation of such loop may control the shuttling of substrate and products to/from the catalytic site

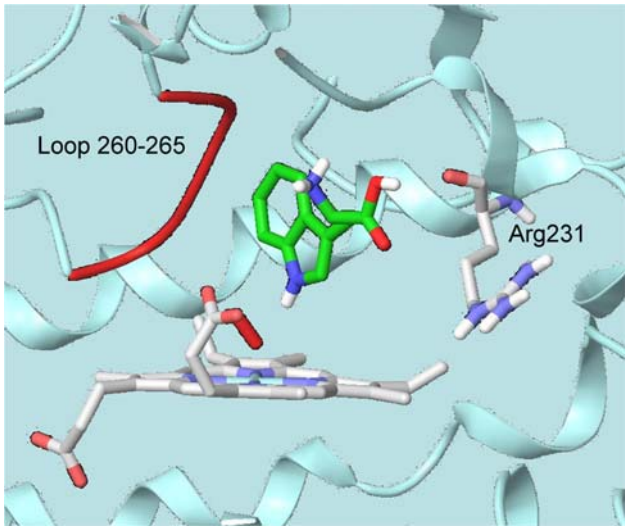


Fig. 5 Proposed binding mode of L-Trp (**1**) into the crystal structure of the ferric form of IDO

pinpointed that mutants Phe226Ala, Phe227Ala and Arg231Ala display a reduced enzymatic activity (Sugimoto et al. 2006). While docking experiments indicate that the latter residue may be directly involved in substrate binding, they further suggest that Phe226 and Phe227 may indirectly affect substrate recognition being involved in π-cation interactions with Arg231. Recently, two single nucleotide polymorphisms (SNPs) have been identified in IDO-2 that suppresses the catalytic activity, namely Tyr239X and Arg248Trp. While the former encodes for a truncated form of IDO-2, the latter corresponds to the key residue Arg231 of IDO suggesting that both enzymatic isoforms might share a common substrate recognition mechanism (Metz et al. 2007).

It should be mentioned that other mutagenesis studies have also involved the residue His303 in the regulation of the catalytic activity of IDO, despite the remote location of such residue in the crystal structure of the enzyme (Papadopoulou et al. 2005).

Interestingly, indole derivatives bearing substituents at 3-position, such as 3-indole-ethanol, have been reported to behave as effectors of IDO by enhancing substrate affinity to the enzyme (Eguchi et al. 1984; Uchida et al. 1985). Kinetic and spectroscopic studies have provided experimental evidences that these compounds bind to an accessory binding pocket located near to the catalytic site of IDO (Sono 1989; Sono and Cady 1989). The inspection of the crystal structures of IDO in complex with 4-phenylimidazole and cyanide (pdb codes: 2D0U, 2D0T) has revealed the presence of a molecule from the crystallization buffer, namely 2-(*N*-cyclohexylamino)ethane sulfonic acid (CHES) that binds in a pocket with the 7-propionate moiety of the heme and the side chains of

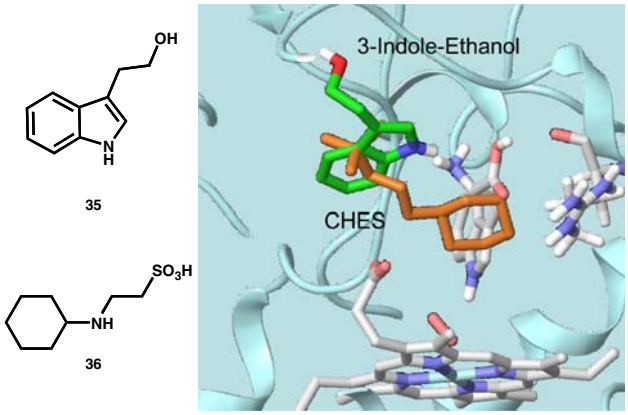


Fig. 6 Proposed binding mode of 3-indole-ethanol (**35**) and observed binding mode of CHES (**36**) into the crystal structure of IDO

Phe226 and Arg231. Docking experiments of 3-indole-ethanol into the crystal structure of IDO provide computational evidences that the pocket occupied by CHES might partially define the allosteric site where the enhancer binds the enzyme (Fig. 6) (Macchiarulo et al. 2007).

The activation cycle

In 1996, Sono and coworkers have proposed a model for the catalytic activation cycle of IDO (Fig. 7) (Sono et al. 1996). The first step of the model is the reduction of ferric state IDO-Fe(III) to the ferrous form IDO-Fe(II), which then binds oxygen and L-Trp (**1**) to form the ternary complex IDO-Fe(II)/oxygen/L-Trp. The authors, however, have not excluded that wherever superoxide is available in the environment, the ferric state of IDO may first bind this reactive oxygen species leading to the formation of a binary complex IDO-Fe(III)/superoxide which, in turn, binds L-Trp (**1**). In this alternative pathway of the activation cycle of IDO, the oxygenated binary complex of the enzyme can be described as a resonance hybrid of IDO-Fe(III)/superoxide and IDO-Fe(II)/oxygen, with the latter being the catalytically active form (Sono et al. 1996 and references therein). In both resonance structures, the binary complex is diamagnetic since there are no unpaired electrons in the Fe(II)/oxygen and the two unpaired electrons are considered to be spin-coupled in Fe(III)/superoxide. The last step of the catalytic cycle is the decomposition of the ternary complex of the enzyme to the ferrous form and the product.

The alternative pathway of the catalytic cycle of IDO, where superoxide plays a major role in the activation of the enzyme, is supported by a great number of experimental evidences. Hayaishi and coworkers have first evidenced the requirement of superoxide and oxygen for the activity of IDO that is inhibited by the addition of superoxide dismutase (Hirata and Hayaishi 1975; Hayaishi et al. 1977; Hirata et al. 1977; Sono et al. 1980). Furthermore, the authors have reported the possible utilization of superoxide by IDO in vivo examining dispersed cell suspensions of the rabbit small intestine and using either inhibitors of superoxide dismutase, or purines as substrates of xanthine oxidase in the presence of methylene blue to enhance the activity of the enzyme (Taniguchi et al. 1977).

Despite these observations, later studies have challenged the notion of superoxide as co-activator and oxygen source for IDO. For instance, investigating the role of superoxide in the presence and absence of methylene blue for the activation of ferric IDO, Sono has pinpointed the importance of methylene blue as an electron donor carrier for the enzyme. Thus, in order to activate IDO under physiological conditions, a specific natural electron carrier would be required in place of methylene blue without demanding superoxide as co-activator. At this aim, reduced flavin mononucleotide (FMNH2) and biopterin (L-5,6,7,8-tetrahydrobiopterin, BH4) have been suggested to accomplish

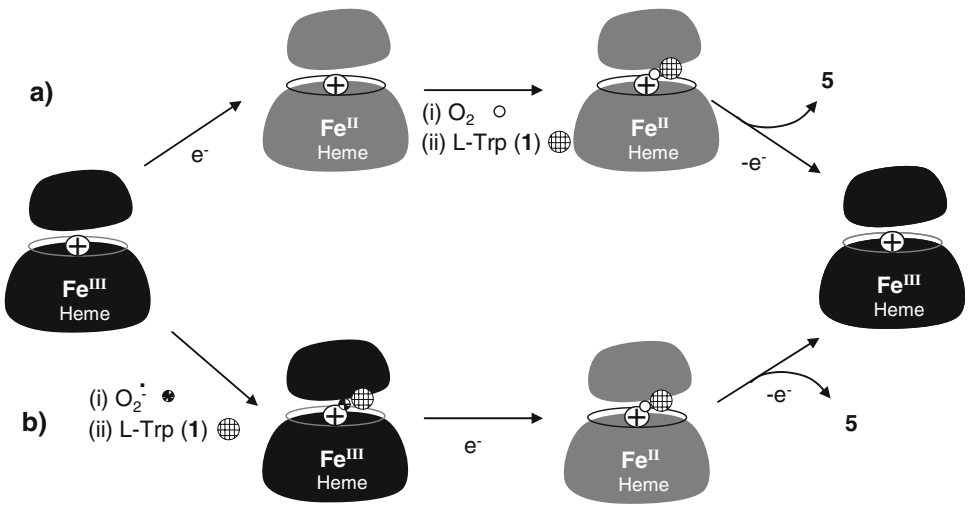


Fig. 7 The proposed canonical (**a**) and alternative (**b**) activation cycle of IDO

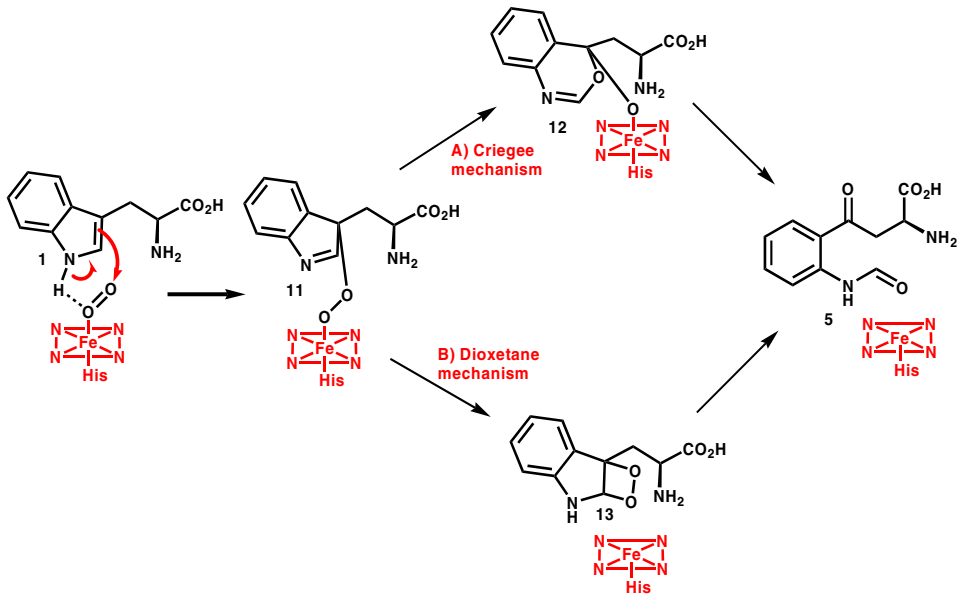


Fig. 8 The proposed catalytic mechanism of IDO

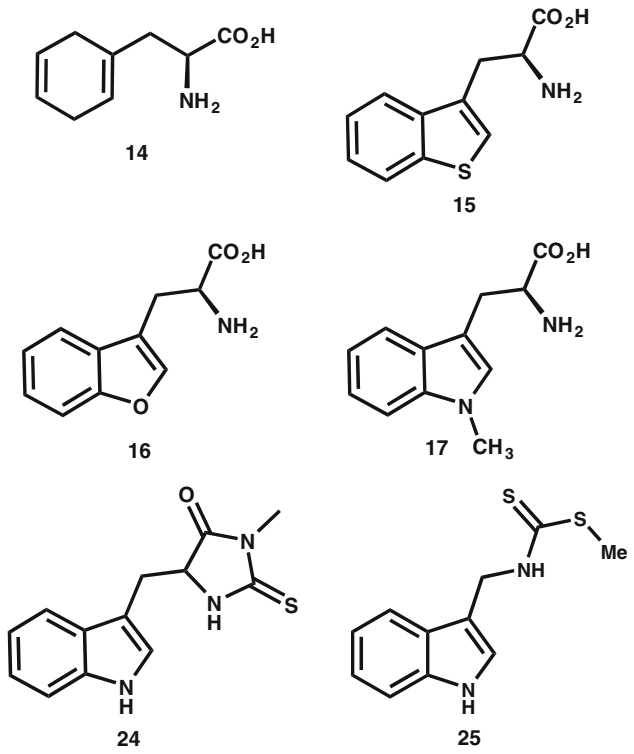


Fig. 9 Competitive inhibitors of IDO

the task being possible electron carriers for IDO in in vitro assays (Ozaki et al. 1986, 1987).

Noteworthy, recent studies have indicated that cytochrome b5 and cytochrome b5 reductase are pivotal factors in maintaining human IDO activity in vivo (Vottero et al. 2006b; Maghzal et al. 2008). Cytohrome b5, in particular, would favor the formation of a transient inter-protein electron transfer (ET) complex (Bendall 1996) with IDO to accomplish the reduction of the latter from the ferric state to the ferrous catalytically active form. In a recent work, Wheeler and coworkers have investigated the molecular basis of the formation of such electron transient complexes using cytochrome b5 and two heme-containing partners, namely myoglobin and hemoglobin, as case studies (Wheeler et al. 2007). The authors have reported the importance of local electrostatic interactions between partner propionates of the heme groups in determining the affinity and lifetime of the ET complexes. In particular, Wheeler and coworkers have dealt with the mechanisms that would establish effective ET complexes while circumventing the large free-energy penalty associated with bringing the negatively charged carboxylates into proximity. The authors have proposed three possible scenarios. In the first one, the propionates do not come in contact and they are bridged by water molecules through hydrogen bonding. A second scenario implies the formation of a ET complex involving the protonation of a propionate group which eliminates the electrostatic repulsion between the negatively charged carboxylate groups. In the last scenario, the propionate group interacts through hydrogen bonds with positively charged residues of the enzyme neutralizing its negative charge. As far as the transient complex between cytohrome b5 and IDO is concerned, the observation of the close proximity of Arg231 to one propionate group of IDO would imply the latter scenario where the basic residue may play a pivotal role in establishing an effective ET pathway for the activation of the enzyme.

Although these studies suggest that the use of superoxide by IDO is insignificant under physiological conditions, it should be mentioned that a recent and worthwhile

observation by Romani and coworkers brings to a new lease of life the notion of superoxide as co-activator of the enzyme under certain conditions. The authors, in particular, have reported the presence of a pathological link between chronic granulomatous disease (CGD), an inherited disorder featured by the lack of NADPH oxidase activity in phagocytes that hampers the production of superoxide, and the impairment of IDO activation which follows the reduced pool of cellular superoxide. Interestingly, the reversal of the hyperinflammatory phenotype of CGD could be achieved by the administration of kynurenine metabolites that reactivated the pathway of tryptophan catabolism downstream the IDO mediated catalytic step (Romani et al. 2008).

Thus, a conclusive consensus evidence on the cellular mechanism involved in the activation step of the catalytic cycle of IDO, namely the reduction of the ferric state IDO-Fe(III) to the ferrous form IDO-Fe(II), is far to be achieved. Notwithstanding, it is possible that depending on the physiological or pathological conditions, cell types, and in vitro or in vivo experiments, all the above cited mechanisms may diversely contribute to the activation of IDO.

The catalytic mechanism

Several reaction mechanisms have been proposed for the oxidative cleavage of the indole nucleus of Trp (Sono et al. 1996). On the basis of the crystal structure and mutagenesis data, Sugimoto and coworkers (Sugimoto et al. 2006) have advanced the hypothesis that the proton abstraction of the indole NH group by iron-bound dioxygen is the most plausible event for the reaction mechanism catalyzed by IDO (Fig. 8). In particular, the authors have indicated that the trigger of such event is the interaction of the indole NH group with the oxygen atom of molecular oxygen bound to the iron-heme. This mechanism has been recently supported by resonance Raman and optical absorption spectroscopies (Batabyal and Yeh 2007).

Then, the reaction proceeds with the electrophilic addition of the iron-bound dioxygen to the double bond at positions C_2–C_3 of the indolic ring that leads to the formation of the 3-indolenylperoxy intermediate. This is finally converted to *N*-formylkynurenine (**5**) either through a Criegee type rearrangement, or by the formation of a dioxetane intermediate. In the former mechanism, the 3-indolenylperoxy intermediate (**11**) rearranges and collapses to the corresponding hemiacetal derivative **12**. Ring opening of **12** gives the *N*-formylkynurenine (**5**). In the latter mechanism, the indolenylperoxy **11** is converted to the dioxetane **13**, which is subjected to a retro 2 + 2 cycloaddition to yield *N*-formylkynurenine (**5**) (Zhang et al. 2007).

Although it is not yet clear whether a Criegee type rearrangement or a dioxetane-mediated mechanism takes place in the catalytic reaction mediated by IDO, recent mutagenesis experiments and molecular modeling studies support that the oxidative opening of the indolic ring occurs through a Criegee mechanism in the case of TDO (Zhang et al. 2007).

Chemical tools

Beside the use of biological tools, the pharmacological and pathophysiological characterization of IDO, as well as its validation as clinically useful target, requires the availability of potent and selective chemical tools. With this aim, while the chemical elaboration around the indole nucleus of L-Trp (**1**) has been an effective strategy to provide potent and competitive IDO inhibitors, the screening of natural and synthetic libraries has provided diverse classes of chemical compounds endowed with different mechanisms of enzymatic inhibition. Although these compounds have been reviewed elsewhere (Malachowski et al. 2005; Muller et al. 2005b), nowadays there is an increasing awareness that the pharmacological data reported for IDO inhibitors may generally suffer from issues related to the use of assays neglecting the existence of two enzymatic isoforms of IDO, namely IDO-1 and the recently discovered IDO-2 (Ball et al. 2007, 2008), or the presence of specie-specific activity of IDO inhibitors. With regard to this latter issue, it has been recently reported that the specie-specific activity of competitive inhibitors such as *S*-1MT (*S*-**17**, Fig. 9) shows different inhibitor potency at the human and mouse IDO with the compound being slightly more active at the former enzyme (K_i = 62 μM vs. K_i = 105 μM).

This result clearly indicates that specie-specific variants of the enzyme may affect the translation of experimental observations from one model to other with implications for the development of clinically useful IDO inhibitors (Austin et al. 2008).

In this part of the review we report some highlights in the field of IDO inhibitors, focusing on their mechanism of inhibition and chemical class.

Competitive inhibitors

In 1978 Watanabe and coworkers identified the first weak and non-selective competitive inhibitor of IDO, namely the aminoacid L-2,5-dihydro-phenylalanine (**14**, Ki = 230 μM, Fig. 9), isolated from *Actinomycetes* (Watanabe et al. 1978).

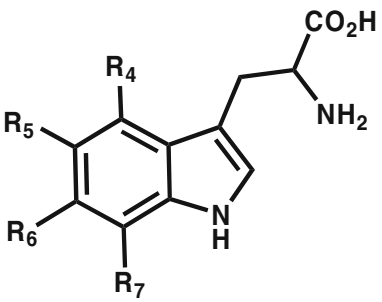
Later on, Cady and Sono reported that analogues of L-Trp (**1**) endowed with the replacement of the indolic

nitrogen with a sulphur atom (benzothiazole derivative **15**) and oxygen atom (benzofuran derivative **16**), or the *N*-methyl substitution at the indolic ring (1-methyltryptophan, 1MT, **17**) were able to inhibit IDO activity with the Ki ranging from low to high micromolar concentration (Cady and Sono 1991). While the racemic mixture of 1MT (**17**) was reported to inhibit IDO with a K_i of 30 μM, different potencies were observed for the *R* (or D) and *S* (or L) isomers, with *S*-**17** (L-1MT) being more potent than the *R*-**17** (D-1MT) in blocking the activity of the enzyme (63 and 12% of inhibition at 100 μM, respectively) (Peterson et al. 1994).

In order to improve the inhibition potency of substrate analogs, electron-withdrawing groups were inserted at positions C-5, C-6, and C-7 of the indolic ring of **1,** with the 5,7-difluoro derivative resulting the most active compound (**23**, Table 1) (Sono et al. 1996; Southan et al. 1996). It should be mentioned, however, that many of these substituted tryptophan derivatives behaved as false substrates of IDO being in part oxidized by the enzyme to yield the corresponding substituted kynurenine derivatives (Malachowski et al. 2005).

Conversely, only few modifications were afforded on the aminoacidic chain. While the esterification of the acidic moiety and the C-alpha or *N*-methylation resulted in poorly active compounds (Peterson et al. 1994; Southan et al. 1996), Muller et al. (2005a) disclosed MTH-Trp (**24**, Fig. 9) as a potent IDO inhibitor ($K_i = 11.4$ μM) by screening commercially available libraries of indole derivatives. It is worth noting that compound **24** features the replacement of the aminoacid moiety of **1** with a thioidantoine ring.

Table 1 Competitive inhibitors of IDO: tryptophan-based analogues

Compound[a]	R4	R5	R6	R7	Inhibition data
					
18		Br			56% at 1 mM
S-19			NO_2		$K_i = 180$ μM
20			F		54% at 1 mM
21				F	$K_i = 37$ μM
S-22	F			F	$K_i = 40$ μM
S-23		F		F	$K_i = 24$ μM

[a] Racemic mixture have been tested unless noted

In a following screening campaign, the same authors identified a natural product, namely brassinin (**25**), as a moderately active competitive inhibitor of IDO with a K_i of about 98 μM (Gaspari et al. 2006). This natural phytoalexin compound bears a methyl dithiocarbamate chain that replaces the aminoacidic chain of L-Trp (**1**).

Further chemical manipulations around **25** allowed the authors to identify a pivotal role for the dithiocarbamic moiety in the inhibition of the enzyme, whereas the indolic ring was not necessary for the activity of these derivatives. Computational studies on the molecular electrostatic potential (MEP) of brassinin analogues suggested a possible binding mode of these compounds where the dithiocarbamic group was involved in a chelating bond with the heme iron of IDO (Gaspari et al. 2006).

Despite the number of competitive IDO inhibitors so far reported, D-1MT (*R*-**17**) has been broadly used in preclinical studies for the proof of concept that IDO inhibition thwarts the tumor immuno-editing process, enhancing the efficacy of current chemotherapeutic agents (Muller et al. 2005b). These observations have prompted clinical trials of D-1MT as drug candidate for anticancer therapy (Muller and Scherle 2006; Hou et al. 2007).

Paradoxically, D-1MT has been recently reported to selectively inhibit IDO-2 (Metz et al. 2007) whereas L-1MT (*S*-**17**) blocks IDO-1. Furthermore, it has been shown that it is IDO-1, and not IDO-2, which is responsible for tryptophan degradation in tumor cells and dendritic cells (Lob et al. 2008a, b). These observations have currently challenged the notion that the therapeutic effect of D-1MT is ascribed to the inhibition of IDO. A number of hypotheses have risen to explain the clinical efficacy of D-1MT (Prendergast 2008). These include the suppositions that the clinical efficacy of D-1MT (*R*-**17**) is attributed to (1) its better pharmacokinetic profile and to the racemization process that occurs in vivo leading to the formation of the active isomer L-1MT; (2) the inhibition of an additional isoform of IDO that is selectively expressed in specific cells; (3) the modulation of a different and still unknown target that mediates the antitumor properties of the compound.

Non competitive inhibitors

β-Carboline derivatives have been the first non competitive inhibitors of IDO described in literature (Fig. 10) (Eguchi et al. 1984; Sono and Cady 1989). Chemical modifications either at the pyridine or phenyl rings of *β*-carboline structure have been instrumental to extend the structure activity relationships of this class of compounds, indicating that the insertion of large alkyl chain in the C-3 position, such as 3-butyl-*β*-carboline (**26**, $K_i = 3$ μM), increases the inhibition potency of the compound (Peterson et al. 1993).

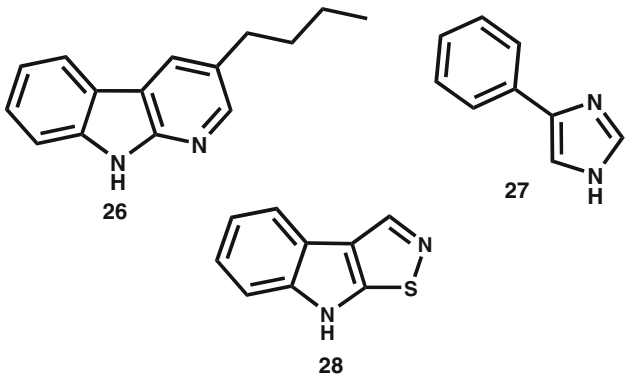


Fig. 10 Non competitive inhibitors of IDO

Other compounds of this class of enzymatic inhibitors include phenyl-imidazole (**27**) and brassilexin (**28**) (Sono and Cady 1989). Non competitive inhibitors of IDO share a common binding mode that features a coordinative interaction involving the electron pair of the basic nitrogen atom and the sixth coordination site of the iron-heme of IDO-Fe(III), as evidenced in the crystal structure of the enzyme with **27** (Sugimoto et al. 2006).

Compounds with uncompetitive and unknown mode of inhibition

Natural products have been fruitful trees of lead compounds endowed with diverse inhibitory activity against IDO. Beside the aforementioned weak inhibitor L-2,5-dihydro-phenylalanine (**14**) and the moderately active compounds brassinin (**25**) and brassilexin (**28**), the screening of natural compounds disclosed Annulin C (**29**, Fig. 11), a marine natural product isolated from the orange hydroid (*Garveia annulata)*, as potent inhibitor of IDO with a K_i of 140 nM (Pereira et al. 2006).

Exiguamine A (**30**), an alkaloid of the marine sponge *Neopetrosia exigua*, was the second marine natural product to be reported as potent IDO inhibitor ($K_i = 210$ nM) (Brastianos et al. 2006).

Efforts toward the reduction of the molecular weight of **30**, led Carr and coworkers to the synthesis of compound **31** having inhibitory activity against IDO comparable to the parent compound (Carr et al. 2008). While an uncompetitive mode of inhibition was claimed for such compound, structure activity relationship (SAR) studies identified the quinone moiety as an essential pharmacophoric element for the inhibitory activity toward the enzyme.

Further screening of natural and synthetic compounds has been recently reported with the identification of naphtoquinone derivatives as novel IDO inhibitors (Kumar et al. 2008). Among the members of this class of compounds, while vitamin K3 (menadione, **32**) has shown an IC_{50} of about 1 μM against human recombinant IDO,

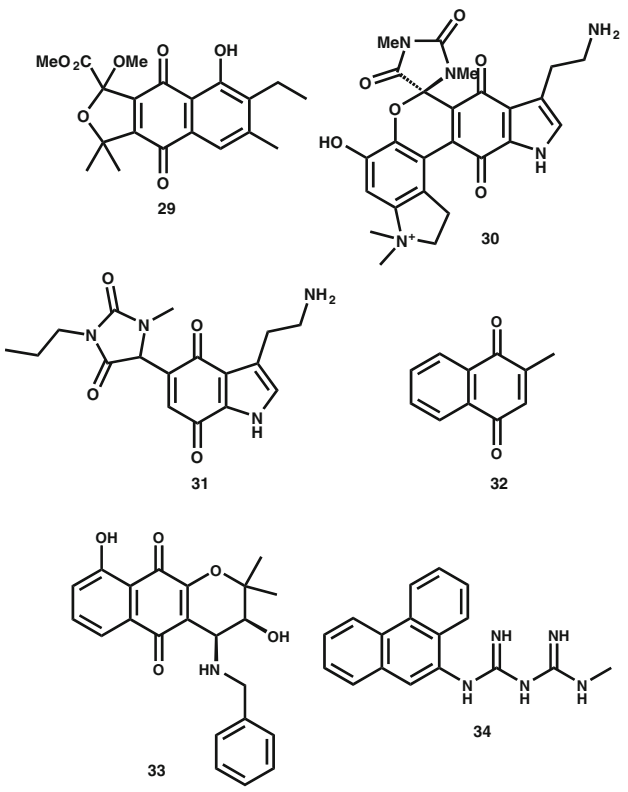


Fig. 11 Compounds with uncompetitive and unknown mode of inhibition

compound **33** proved to be one of the most potent inhibitor of IDO so far available ($K_i = 66$ nM). As well as for compound **31**, an uncompetitive mode of inhibition against IDO has been claimed for this class of compounds. Since naphtoquinone derivatives are featured by the presence of a quinone moiety, the latter observation sustains the key role of this pharmacophoric element in the SAR of uncompetitive inhibitors of IDO suggesting that the oxidation potential of the quinone group may be directly involved in the mechanism of inhibition of such compounds (Kumar et al. 2008).

The screening of synthetic compounds unveiled additional non indolic structures endowed with an unknown mode of inhibition against IDO. Among these, *N*-methyl-*N*′-9-phenanthrenyl-imidodicarbonimic diamide (**34**) is a potent inhibitor of human recombinant IDO with a K_i of 1.5 μM (Vottero et al. 2006a).

Concluding remarks

The scientific interest in IDO is unceasingly increasing ever since the enzyme was reported to be involved in the mechanisms of immune tolerance and tumor immunoediting. These observations have indeed shed new light on

IDO as novel therapeutic target for the development of new cancer drugs. Although many of the efforts in IDO research have been directed toward the pathophysiological characterization of the enzyme, its validation as clinically useful target, and the elucidation of its structural and mechanistic aspects, these studies have raised new questions that need to be addressed by further investigations. In particular, clarifying the roles of different isoforms of IDO in the aforementioned mechanisms that have been indiscriminately attributed to only one enzyme, and the elucidation of the cellular mechanisms responsible for the activation and regulation of IDO, will be two of the next tasks in this field of research.

The availability of new potent and selective inhibitors of both IDO and IDO-2 will prove pivotal to their accomplishment. To this end, it is likely that the future trends of IDO research will see new endeavors toward the identification of structurally different classes of inhibitors and the optimization of the current chemical tools that, while paving the way to the development of novel drug candidates for cancer therapy, will complete the unraveling of the functional and mechanistic aspects of the enzyme filling the gaps of the current knowledge on IDO.

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