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MINIREVIEW ARTICLE

Indoleamine 2,3-dioxygenase 2 (IDO2) and the kynurenine pathway: characteristics and potential roles in health and disease

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Abstract The kynurenine pathway is the major route for the oxidative degradation of the amino acid tryptophan. Activity of the pathway is involved in several disease conditions, both in the periphery and the central nervous system, including cancer, inflammatory disorders, neurological conditions, psychiatric disorders and neurodegenerative diseases. Three enzymes are now known to catalyze the first and rate-limiting step in the catabolism of tryptophan along this pathway: tryptophan 2,3-dioxygenase (TDO) and indoleamine 2,3-dioxygenase (IDO, subsequently named IDO1), both of which have been extensively studied, and a third enzyme, indoleamine 2,3-dioxygenase 2 (IDO2), a relative newcomer to the kynurenine pathway field. The adjuvant chemotherapeutic agent, 1-methyl-D-tryptophan, was intially suggested to target IDO2, implying involvement of IDO2 in tumorigenesis. Subsequently this compound has been suggested to have alternative actions and the physiological and pathophysiological roles of IDO2 are unclear. Targeted genetic interventions and selective inhibitors provide approaches for investigating the biology of IDO2. This review focuses on the current knowledge of IDO2 biology and discusses tools that will assist in further characterizing the enzymes of the kynurenine pathway.

Keywords Kynurenine pathway · Tryptophan · Indoleamine 2,3-dioxygenase · Tryptophan 2,3-dioxygenase · 1-Methyltryptophan

Abbreviations

CNS	Central nervous system
IDO	Indoleamine 2,3-dioxygenase
IFN-γ	Interferon-γ
LIP	Liver-enriched inhibitory protein
MB	Methylene blue
1DMT	1-Methyl-D-tryptophan
1LMT	1-Methyl-L-tryptophan
1MT	1-Methyltryptophan
NMDA	*N*-Methyl-D-aspartate
NAD^+	Nicotinamide adenine dinucleotide
rmIDO	Recombinant mouse indoleamine 2,3-dioxygenase
rhIDO	Recombinant human indoleamine 2,3-dioxygenase
TDO	Tryptophan 2,3-dioxygenase
L-Trp	L-Tryptophan

Introduction

L-Tryptophan (L-Trp) is one of the essential amino acids and is the least abundant of all dietary amino acids. It is highly hydrophobic and possesses an indole ring attached to a methylene group. Apart from its role in protein synthesis, L-Trp is utilized in other physiological processes generating biologically active products, including the biosyntheses of the aminergic neurotransmitter serotonin (5-hydroxytryptamine, 5-HT), the neurohormone melatonin

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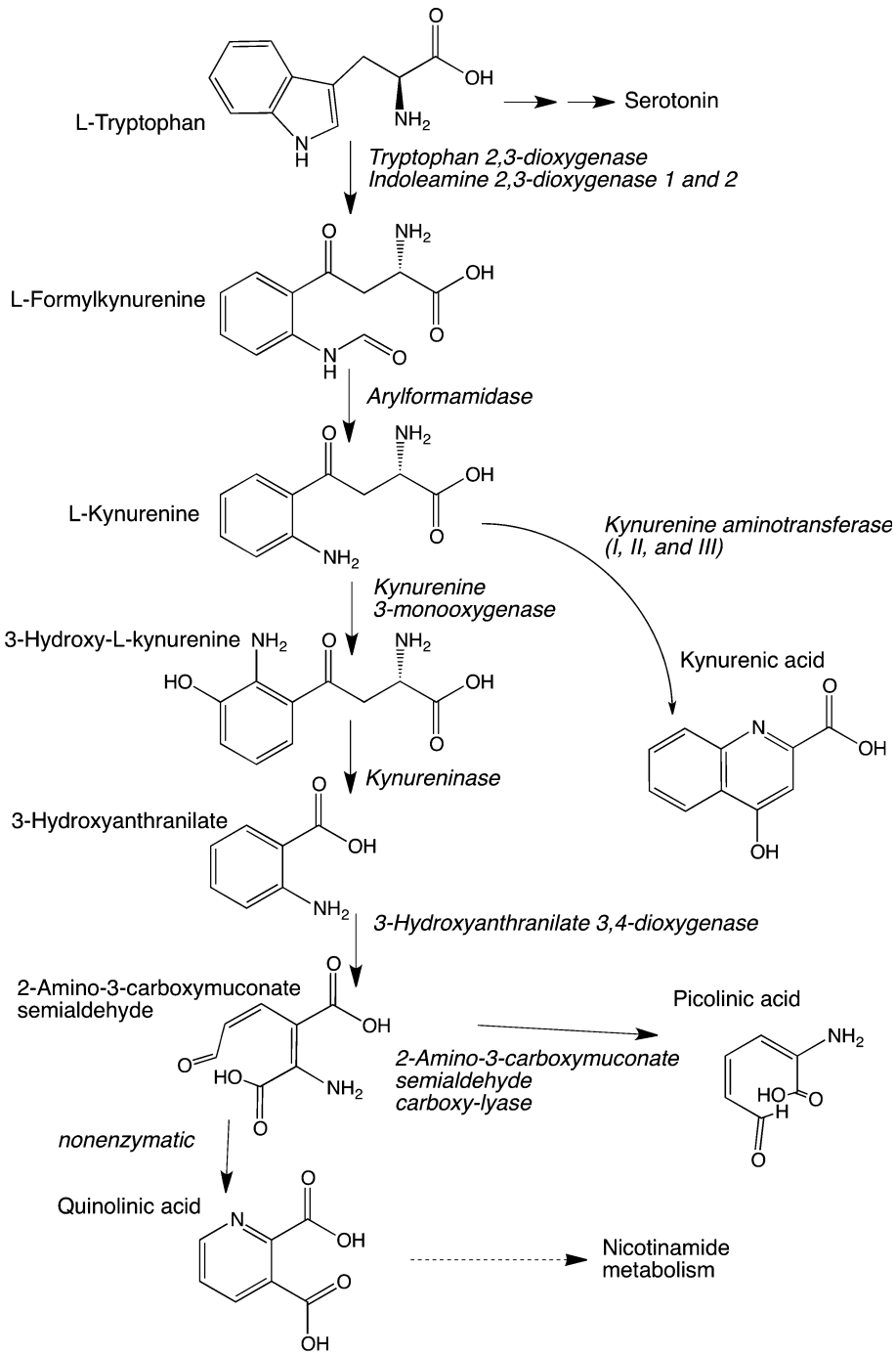

Fig. 1 The kynurenine pathway of tryptophan metabolism

and its kynuramine metabolites, and the trace amine tryptamine, as well as products of the kynurenine pathway. Most dietary Trp is metabolized through the kynurenine pathway (Leklem 1971). Activation of the kynurenine pathway produces several downstream intermediates in a multi-stage and branched arrangement, mediated by enzymes acting at the different levels of the cascade. At the end of the oxidative pathway appears nicotinamide adenine dinucleotide (NAD^+), a co-factor required in many biochemical processes that can also be supplied via dietary niacin (Beadle et al. 1947). The kynurenine pathway is a major route for NAD^+ synthesis (Fig. 1), and in some mammals can supply sufficient NAD^+ in the absence of any dietary niacin intake (Axelrod et al. 1945).

Over the past few decades, the kynurenine pathway has emerged as a major research focus. Broadly speaking, activity of the pathway has two effects: depletion of L-Trp and production of biologically active metabolites. The depletion of L-Trp can have a significant negative effect on cell proliferation, particularly that of *T* cells.

Immunomodulation by L-Trp depletion can thus influence the tolerance of tumors, transplants and the allogeneic fetus (Alexander et al. 2002; Munn et al. 1998; Uyttenhove et al. 2003). Lack of the essential amino acid can also suppress the growth of microbes, so activation of the pathway can be an antimicrobial mechanism (Pfefferkorn and Guyre 1984). In addition, the products of the pathway have wide-ranging, sometimes opposing, effects, especially in the brain. Some of these products have neuroactive (detrimental or protective) properties, for example, quinolinic acid, an agonist that activates glutamate receptors sensitive to *N*-methyl-D-aspartate (NMDA) (Perkins and Stone 1983), leading to excitotoxicity (Schwarcz et al. 1983). In contrast, kynurenic acid is an antagonist at the NMDA and other glutamate receptors (Perkins and Stone 1982) and also inhibits alpha7 nicotinic receptor activity (Hilmas et al. 2001). Several kynurenine pathway products, in particular 3-hydroxykynurenine and 3-hydroxyanthranilic acid, have antioxidant activity (Christen et al. 1990). Thus, activation of the pathway may be neurotoxic or neuroprotective (Hunt et al. 2006).

The pathway has been implicated in many disorders and/or their complications, including cerebral malaria (Sanni et al. 1998), neurological and neurodegenerative diseases (Darlington et al. 2007; Guidetti et al. 2000; Guillemin et al. 2003; Ogawa et al. 1992), acquired immune deficiency syndrome-related dementia (Heyes et al. 1989), rheumatoid and osteo-arthritis (Criado et al. 2009; Igari et al. 1975), chronic granulomatous disease (Romani et al. 2008) and sepsis (Changsirivathanathamrong et al. 2011). Therapeutic modulation of the pathway in these diseases is still a distant prospect. The exception is cancer where a modulator of kynurenine pathway activity, 1-methyl-D-tryptophan (1DMT), is in clinical trials. This review will explore this field, in the context of a recently discovered enzyme of the pathway, indoleamine 2,3-dioxygenase-2 (IDO2), an enzyme once suggested to be the target of 1DMT. We will outline the current understanding of IDO2 biology and the prospects for modulating its activity.

Indoleamine 2,3-dioxygenase 1 (IDO1) and Tryptophan 2,3-dioxygenase (TDO)

Although many enzymes participate in the kynurenine pathway from initiation to completion, indoleamine 2,3-dioxygenase (IDO) (EC 1.13.11.52) and TDO (EC 1.13.11.11) are the only enzymes responsible for the first and rate-limiting reaction. The cleavage of the 2,3-double bond of the indole ring of Trp, through the incorporation of molecular oxygen, leads to the formation of *N*-formylkynurenine, which is rapidly converted, through deformylation, to L-kynurenine (Hayaishi et al. 1957; Heidelberger et al. 1949). IDO is a monomeric, heme-containing enzyme (Shimizu et al. 1978). It is active when the heme iron is in the ferrous form (Fe^{2+}) but inactive when it is in the ferric form (Fe^{3+}), and its substrate inhibition could be linked to the binding of Trp to the ferric form (Sono et al. 1980). Until recently, only one form of IDO was known, which has now necessarily been renamed IDO1, to distinguish it from the newly identified isoform, IDO2. IDO1 and TDO both contain heme but differ in their substrate specificities and tissue expression patterns. The monomeric IDO1 has a broader substrate range than the homotetrameric TDO, capable of cleaving D- and L-Trp, tryptamine, 5-hydroxytryptophan and 5-hydroxytryptamine (Shimizu et al. 1978). In fact, it was initially isolated from rabbit intestine as a D-Trp-degrading enzyme (Yamamoto and Hayaishi 1967). In mammals, TDO is highly expressed in the liver, where it is induced by glucocorticoids and L-Trp (Knox 1951; Knox and Auerbach 1955), and is also found in the brain (Haber et al. 1993). Notably, TDO, which is enantiomer-specific (can only cleave L-Trp), has been suggested to be the key regulatory enzyme in modulating circulating L-Trp levels (Kanai et al. 2009) and is believed to have a major role in supplying NAD^+ through the kynurenine pathway (Badawy 1981).

IDO1 expression is found in most tissues and is regulated by immunological signals, including interferon-γ (IFN-γ), lipopolysaccharide and tumor necrosis factor (Pfefferkorn and Guyre 1984; Werner-Felmayer et al. 1990; Yoshida and Hayaishi 1978). In mammals, it is recognized as a defence molecule in combating bacterial and viral infections, as its expression is up-regulated by cytokines such as IFN-γ, leading to local depletion of L-Trp and causing inhibition of pathogen growth (Byrne et al. 1986). The immunomodulatory roles of IDO1 also have been highlighted by the recognition that its pharmacological inhibition caused the rejection of mouse allogeneic concepti, mediated by *T* cells (Munn et al. 1998), and its expression in tumors is associated with their immune evasion (Friberg et al. 2002; Uyttenhove et al. 2003).

Identification of IDO2

In 2007 a gene with homology to IDO was reported (Murray 2007), following which three independent groups showed that an enzyme with the ability to degrade Trp was encoded by this gene (Ball et al. 2007; Metz et al. 2007; Yuasa et al. 2007). A variety of names has been given to this enzyme, including indoleamine 2,3-dioxygenase-like protein, indoleamine 2,3-dioxygenase-2 (IDO-2 or IDO2) and proto-indoleamine 2,3-dioxygenase (proto-IDO). It is now most commonly referred to as IDO2 and has a protein size of 420 amino acids (≈47 kDa) and 398 amino acids

(≈45 kDa), in humans and mice, respectively. Although our knowledge of this new enzyme is growing, little is still known, in comparison to the more familiar IDO1, about its exact physiological roles. In humans and mice, IDO1 and IDO2 proteins have 43 % homology at the amino acid level, but very little homology with the TDO protein (Ball et al. 2007). When their sequences were aligned, residues necessary for catalytic activity, as determined by IDO mutagenesis and crystallographic analysis, were highly conserved in IDO2 (Metz et al. 2007). The genes have a similar genomic structure and are situated adjacent to each other on chromosome 8 in humans and mice (Ball et al. 2007; Yuasa et al. 2007). It is most likely that the genes encoding these proteins emerged through gene duplication in a mammalian ancestor after the divergence of the mammalian lineage, as only mammalian genomes have been found to encode both IDO proteins (Yuasa et al. 2009). Lower vertebrates possess one *IDO*, more similar to *IDO2* than *IDO1* in sequence, suggesting that the ancestral gene may be closer to *IDO2* (Yuasa et al. 2010). The proximity of both *IDO* genes has been suggested to predict a potentially coordinated regulation. Consistent with this view, a significant down-regulation of the *IDO2* transcript was found in some tissues from an *IDO1* gene knockout mouse strain, which could be due to the loss of regions of the *IDO1* gene leading to disruption of the regulatory elements of the neighboring *IDO2* gene (Ball et al. 2007).

IDO2 expression

Transcription of the *IDO2* gene is complex and it is difficult to assess the expression of a functional enzyme simply by amplifying part of the transcript. For example, the expression of at least two transcripts with alternative 5′exons suggests that the mouse gene has two promoter regions and only one of these transcripts would encode full-length protein (Ball et al. 2009). In addition, there is the existence of several alternatively spliced transcripts for human IDO2, with one alternative transcript for the human *IDO2* gene, carrying an extra thirteen amino acids, showing a more restricted tissue expression (Metz et al. 2007). The longer human IDO2 isoform was reported to have no enzymatic activity in an in vitro assay (Meininger et al. 2011).

Studies combining mRNA and protein analysis suggest that mouse IDO2 is expressed in the liver, epididymis and kidney (Ball et al. 2007; Fukunaga et al. 2012). There is some disparity as to whether IDO2 is expressed in the mouse brain as well as to its distribution in the male reproductive system. These patterns will be further clarified with the development of more IDO2 antibodies and the assessment of their specificity using $IDO2^{-/-}$ mice. It is difficult to assess the distribution of IDO2 in humans as so few studies have examined both protein and mRNA expression. IDO2 mRNA and protein expression has been detected in pancreatic cancer cell lines (Witkiewicz et al. 2009). In addition, *IDO2* mRNA has been detected in gastric, colon and renal tumors (Lob et al. 2009).

A striking feature of IDO1 expression is its induction by IFN-γ, therefore the regulation of IDO2 expression by this cytokine has also been examined. Interestingly, *IDO2* mRNA expression was upregulated in response to IFN-γ in some cancer cell lines (Lob et al. 2009; Witkiewicz et al. 2009). This also has been reported for *IDO2* mRNA in mouse dendritic cell lines (Metz et al. 2007; Sun et al. 2010), as well as mouse and human mesenchymal stem cells (Croitoru-Lamoury et al. 2011). *IDO2* mRNA was also upregulated in the brain of mice infected with *Toxoplasma gondii*, an infection in which IFN-γ-driven responses play an important role in controlling parasite growth (Divanovic et al. 2012). *IDO2* mRNA levels were induced by IFN-γ in HeLa cells (Lob et al. 2009). However, in another study, *IDO2* mRNA levels were not altered in HeLa cells treated with IFN-γ, even though *IDO1* mRNA was strongly induced (Liu et al. 2010). These opposing findings may be due to the complexity of *IDO2* transcripts and underline the need for corresponding measurements of IDO2 protein expression. In an experimental cerebral malaria model, a disease with high-circulating levels of IFN-γ, IDO2 mRNA and protein expression were not observed to be upregulated (Ball et al. 2007).

Functional and biochemical comparison of IDO1 and IDO2

The first descriptions of IDO2 showed that the mouse enzyme, when transfected into mammalian cells, was able to catabolize L-Trp to form kynurenine, although it seemed to possess lower enzymatic activity than mouse IDO1 (Ball et al. 2007; Metz et al. 2007). L-Trp depletion activates a signaling pathway including phosphorylation of the translation initiation factor eIF-2α and translation of liver-enriched inhibitory protein. Both IDO1 and IDO2 activity were sufficient to induce this signaling pathway, although IDO2 produced a distinct signal for liver-enriched inhibitory protein activation that was independent of L-Trp availability, whereas the signal produced by IDO1 was dependent on the availability of L-Trp (Metz et al. 2007). Activity of the kynurenine pathway also supplies NAD^+ and deletion of the only IDO in a yeast strain renders it auxotrophic for nicotinic acid, an alternative source of NAD^+. Replacing the yeast IDO with mouse IDO1 or mouse IDO2 showed that mouse IDO1 activity is sufficient to rescue the mutant strain; however, mouse IDO2 does not have this capacity (Yuasa and Ball 2013). The higher

affinity of L-Trp for the IDO1 isoform relative to the IDO2 isoform has also been observed in other mammals, such as monotremes and marsupials (Yuasa et al. 2009).

It is uncertain whether human IDO2 can function as a L-Trp catabolizing enzyme based on its low enzymatic activity. Some studies were not able to detect appreciable formation of kynurenine in HEK293 cells transiently transfected with human IDO2 (Liu et al. 2010; Qian et al. 2009; Yuasa et al. 2010). Another study was able to measure human IDO2 activity in T-REX cells derived from the HEK293 cell line (Metz et al. 2007), suggesting that the activity of human IDO2 may be influenced by the stable expression of the protein or differences in culture conditions. Human IDO2 expression is not able to rescue a yeast strain auxotrophic for nicotinic acid, suggesting it does not have sufficient activity to supply NAD^+ in yeast (Yuasa and Ball 2013). Human dendritic cells express both *IDO1* and *IDO2* mRNA, although IDO2 protein expression has not been demonstrated (Lob et al. 2008). An IDO1-selective inhibitor blocked kynurenine formation completely in human dendritic cells, suggesting that IDO1 was responsible for L-Trp metabolism in the cells (Liu et al. 2010). Furthermore, inhibiting IDO1 expression through siRNA also effectively blocked kynurenine formation in stimulated human dendritic cells and cancer cell lines, further indicating that IDO2 is not enzymatically active in these cells (Lob et al. 2008, 2009). The transcriptional complexity of the *IDO2* gene and the high population frequency of two genetic polymorphisms in the coding sequence mean that activity studies on the endogenous enzyme are difficult to interpret without data on protein expression and genotype. However, the current studies do raise several possibilities: IDO2 in humans has evolved to be a biologically inactive protein, human IDO2 has a role that is independent of enzymatic activity, or the enzyme is active under conditions that have not been well-characterized as yet. The concept of an alternative activity for IDO2 is consistent with the recent finding of a signaling role for the IDO1 protein that is independent of enzymatic activity (Pallotta et al. 2011). Although IDO2 was not found to be involved in the same signaling pathway, it does demonstrate that the IDO enzymes can possess functions apart from L-Trp metabolism. The final possibility, that human IDO2 is active under specific conditions, is again supported by indirect evidence. Culture of human basal carcinoma cells with the chemokine CXCL11 induced kynurenine formation (Lo et al. 2011). This correlated with the induction of human *IDO2*, but not *IDO1*, mRNA expression, suggesting IDO2 was responsible for the kynurenine formation. It is possible that IDO2 activity is determined by the presence of particular co-factors and is only evident in certain cell types or conditions.

The distinct requirements of IDO2 for enzymatic activity are demonstrated by studies using in vitro assays. Kinetic activity of IDO enzymes is generally determined using the methylene blue (MB)/ascorbic acid assay, with MB serving as the electron source (Takikawa et al. 1988). For many years, superoxide anion was thought to be the cellular reductant of IDO1, but it now seems more likely that cytochrome b_5 is the major physiological electron donor (Maghzal et al. 2008; Vottero et al. 2006). Human IDO1 was active in an in vitro assay containing cytochrome b_5, cytochrome P450 reductase and an NADPH-regenerating system, although it showed greater activity in the traditional MB assay (Maghzal et al. 2008). In contrast, recombinant mouse IDO2 (rmIDO2) and human IDO2 showed very low activity in the assay containing MB (Austin et al. 2010; Meininger et al. 2011). The use of the cytochrome b_5 assay reduced the K_m of rmIDO2 by 20-fold and increased the V_{max} by 4.5-fold, compared to the MB assay (Austin et al. 2010). The cytochrome b_5/cytochrome P450 reductase system may act to positively modify IDO2 activity either through direct electron transfer or through modification of the active site by way of a two-heme protein complex, thus increasing substrate access or decreasing uncoupling time of product (Austin et al. 2010). Some other features of the recombinant mouse IDO proteins are summarized in Table 1.

IDO inhibitors

IDO inhibitors have been employed to uncover the role of the kynurenine pathway in physiological processes. For example, inhibition of IDO activity with 1-methyltryptophan (1MT) resulted in the rejection of the allogeneic fetus, indicating a role for IDO activity in tolerance (Munn et al. 1998). The immunomodulatory role of IDO has also suggested a therapeutic potential for IDO inhibitors, specifically as an adjuvant chemotherapy. IDO activity has been associated with suppressing the immune response towards

Table 1 Biochemical characteristics of mouse IDO1 ad IDO2 enzymes

	Mouse IDO1	Mouse IDO2
Optimal pH[a]	6.0–6.5	7.4–7.5
Substrate inhibition (K_i)[b]	1.6 mM	None at >50 mM
Denaturation (T_m)[a]	60 °C (major), 48 °C (minor)	48 °C
K_m MB assay[a]	28 ± 4 μM	12,000 ± 3,000 μM
K_m Cytochrome b5 assay[a]	29 ± 9 μM	530 ± 100 μM

[a] Austin et al. 2010

[b] Yuasa et al. 2009

tumor cells (Friberg et al. 2002; Uyttenhove et al. 2003). Past studies employing the 1MT as an inhibitor have assumed that IDO1 was its only target, as the existence of IDO2 was unknown at the time.

Historically, both chemical elaboration around the indole nucleus of L-Trp and screening of natural and synthetic libraries have yielded potent and competitive or non-competitive IDO1 inhibitors. Some have known mechanisms of action but for others there is as yet no equivalent understanding. Derivatives of L-Trp have provided a number of inhibitors, although their mode of action might also be described as "slow substrates" (Chauhan et al. 2009). The most widely used of these analogs is 1MT, which has an *N*-methyl substitution at the indolic ring (Cady and Sono 1991). The stereoisomers of 1MT, 1-methyl-L-tryptophan (1LMT) and 1DMT exhibit different potencies for rmIDO1 inhibition, with the K_i for 1DMT being 100-fold higher than that for 1LMT (Austin et al. 2010). IDO1 inhibitors have been reviewed by others (Macchiarulo et al. 2009).

Resolving the 1MT conundrum

Numerous studies have associated IDO1 expression in tumors or within dendritic cells of tumor-draining nodes with a poorer prognosis (reviewed by Godin-Ethier et al. 2011). Administration of 1MT enhanced the immune-mediated rejection of tumors in a mouse model (Uyttenhove et al. 2003). Consistent with this, reduced tumor growth was observed in vivo when siRNA was used to silence the *IDO1* gene in B16F10 tumor-bearing mice (Zheng et al. 2006). Testing of the 1MT stereoisomers revealed that 1DMT was more effective in abrogating the inhibition of *T* cell proliferation in mixed lymphocyte reactions and in reducing the growth of tumors in mouse models (Hou et al. 2007). This was surprising as 1LMT was a better pharmacological inhibitor of IDO1 than 1DMT in both cell-free and cellular assays (Hou et al. 2007; Peterson et al. 1994). The greater in vivo efficacy of 1DMT has led to this stereoisomer being used for adjuvant chemotherapy in clinical trials. One hypothesis for the efficacy of 1DMT was that it targets an additional IDO1 isoform selectively expressed in specific cells (Hou et al. 2007). The suggestion of an alternative target for 1DMT was further supported by the discovery of IDO2. One of the initial reports of the existence of IDO2 showed that it was selectively inhibited by 1DMT over 1LMT (Metz et al. 2007). This raised the possibility that IDO2 activity was involved in the evasion of an immune response by tumors. However, it was found that 1DMT was not efficacious in a tumor model on an $IDO1^{-/-}$ background, suggesting that its effects were mediated through IDO1 (Hou et al. 2007). The expression of IDO2 protein in the liver and kidney in these $IDO1^{-/-}$ mice is unaffected, although the *IDO2* transcript is downregulated (Ball et al. 2007). It is possible that the deletion of the *IDO1* gene affects the expression of the IDO2 protein in other cell types, and 1DMT's effects might be mediated through either or both IDO isoforms.

Further studies have found that 1LMT is a better inhibitor of IDO2 enzymatic activity than 1DMT in both cell-free and cellular assays (Austin et al. 2010; Qian et al. 2012; Yuasa et al. 2010). It is possible that 1DMT is an IDO2-selective inhibitor under certain conditions (Metz et al. 2007), but in most studies 1LMT is the more effective inhibitor of Trp depletion by either IDO1 or IDO2 (Austin et al. 2010; Lob et al. 2008; Qian et al. 2012; Yuasa et al. 2010). An alternative explanation for the in vivo efficacy of D1MT as an adjuvant chemotherapy has been proposed in a recent study. Trp depletion by the kynurenine pathway activity activates the stress response kinase GCN2, thereby triggering a signaling pathway leading to suppression of *T* cell proliferation (Munn et al. 2005). Although GCN2 kinase activation was involved in IDO1-mediated *T* cell suppression, its absence did not have an effect on tumor progression (Metz et al. 2012; Munn et al. 2005). Trp depletion was shown to modulate another signaling pathway, namely inhibition of mTOR, a sensor of amino acid sufficiency. Repression of mTOR activity leads to autophagy; however, 1DMT acted as a Trp mimetic, providing an amino acid sufficiency signal that prevented autophagy (Metz et al. 2012). This provides an alternative mechanism for the efficacy of 1DMT in inhibiting tumor progression, in the light of its poor inhibition of IDO1 and IDO2 enzymatic activity observed in other assays. As TDO activity also has been implicated in the inhibition of immune responses against tumors (Pilotte et al. 2012), a molecule acting downstream of all three Trp-catabolizing enzymes may have more potential as a therapeutic agent.

The mTOR pathway may explain the efficacy of 1DMT in tumorigenesis, but 1DMT also has been used in models where this pathway would not be expected to play a role. Kynurenine was shown to be a vascular-relaxing agent produced by IDO1 expression in endothelial cells during systemic inflammation (Wang et al. 2010). 1DMT was shown to both inhibit kynurenine formation in isolated porcine coronary arteries as well as reduce a drop in blood pressure observed in systemic inflammation (Wang et al. 2010). This suggests that 1DMT has effects in addition to interacting with the mTOR pathway. It has been suggested that reductants modulate inhibitor binding and activity (Metz et al. 2012) so IDO inhibition by 1DMT might only be observed in cell types with a particular mix of cofactors/reductants.

Understanding the role of IDO2: the way forward

As the kynurenine pathway has been implicated in a myriad of pathological conditions, the discovery of IDO2 represents a new field of study and potentially a target for therapeutics development. Experimentally, investigating the biological functions of enzymes employs strategies including gene manipulation (gene silencing through the use of siRNAs, transgenic or knock-out technologies) and the use of selective, small-molecule inhibitors. To date, siRNA approaches have mostly been used in cell lines and primary cells (Lob et al. 2008; 2009; Pallotta et al. 2011; Zheng et al. 2006) but have also successfully been employed in an in vivo model to reduce IDO1 activity (Zheng et al. 2006). *IDO1*-null and *TDO*-null mice have been described (Baban et al. 2004; Kanai et al. 2009) and *IDO2*-null mice also exist, although are not described in the literature as yet. *TDO*-null mice have greatly increased circulating levels of Trp, leading to increased serotonin biosynthesis and alterations in behavior and neurogenesis, suggested to be the result of changes to serotonin metabolism (Kanai et al. 2009). TDO is likely to be a key regulator of tryptophan homeostasis in mammals through its enzymatic activity in the liver.

IDO1 is constitutively expressed in the epididymis and *Ido1*-deficient mice have increased inflammatory markers in the epididymis and abnormal spermatozoa, although they remain fertile (Jrad-Lamine et al. 2011). As IDO1 expression is also inducible, many of the differences between IDO1-deficient and expressing mice are not observed until the mice undergo some kind of challenge. For example, IDO1 deficiency resulted in more severe inflammation-associated pathologies, including increased severity of disease in an arthritis model, incidence of pancreatitis in response to adjuvant administration and incidence of colon tumors following colitis (Chang et al. 2011; Criado et al. 2009). Interpretation of the $IDO1^{-/-}$ phenotype is complicated by two factors. Deletion of *IDO1* genomic sequences has the potential to also impact on IDO2 expression due to the chromosomal proximity of the genes. Indeed, transcription of the *IDO2* gene is reduced in the liver of $IDO1^{-/-}$ mice, although protein levels appear to be maintained (Ball et al. 2007). Further investigations of these mice might also examine IDO2 expression in other cell types. Second, a disadvantage of gene knockout technology is that, during development, compensatory changes or developmental defects may make interpretation of the phenotype complex. IDO inhibition with 1MT resulted in the rejection of allogeneic concepti; however, $IDO1^{-/-}$ mice will tolerate allogeneic concepti (Baban et al. 2004; Munn et al. 1998). The use of 1MT in the $IDO1^{-/-}$ mice did not result in rejection, therefore it is still probable that the 1MT's effects are mediated through modulating kynurenine pathway activity (Baban et al. 2004). Studies using both short-term (transient overexpression, siRNA knockdown or pharmacological inhibition) and long-term (transgenic expression or gene deletion) modulation of enzymatic activity will separate the effects of enzymatic activity from non-specific effects or compensatory/developmental changes. In addition, double knockout mice will be useful in understanding which facets of the Trp catabolism remain with only one of the three enzymes present. One caveat for using mice as a model for understanding the role of IDO2 in humans is the lower enzymatic activity of human IDO2. Commonly occurring genetic polymorphisms reduce this activity even further and gene association studies may have a role in linking human IDO2 to physiological phenotypes or diseases (Metz et al. 2007).

Small molecule inhibitors can have a number of advantages over gene manipulation technology to study enzyme function. It is possible to block the target in a reversible fashion and graded responses can be examined through the use of different concentrations or doses of the small molecule. They also have potential as therapeutic agents, as suggested by the link of the *IDO2* polymorphisms with responses to a chemotherapeutic agent for metastatic brain tumors (Eldredge et al. 2012). Optimal IDO inhibitors would be small molecules that can distinguish between the two isoforms, preferably at low nanomolar range of concentrations, and target each while largely avoiding non-IDO targets. At present, two compounds (INCB024360 and Amg-1) selective for IDO1 inhibition over IDO2 inhibition have been reported (Liu et al. 2010; Meininger et al. 2011). In addition, a compound, 680C91, was found to be selective for TDO inhibition over IDO1 inhibition (Pilotte et al. 2012). These inhibitors, in addition to IDO2-selective inhibitors, will be useful in defining the role of each enzyme.

Identifying IDO2-selective inhibitors presents some challenges. First, as IDO1 and IDO2 share significant structural similarity and utilize the same substrate, it might be expected that some compounds also interact with the binding site for both enzymes. Accordingly, 1LMT was shown to inhibit both IDO1 and IDO2 activity (Austin et al. 2010; Qian et al. 2012; Yuasa et al. 2010). Understanding the structure of the two enzymes, through modeling or experimental studies, may allow the design of molecules that discriminate between IDO1 and IDO2 binding sites. These compounds would still need to be tested in experimental assays to validate the predictions. Finding the optimal assay for IDO2 activity, whether for testing candidate compounds or screening libraries, is another challenge. Clearly the MB/ascorbic acid assay is not optimal for measuring IDO2 activity and the cell-free assay containing cytochrome b_5 is expensive for screening purposes (Austin et al. 2010). A cellular-based assay provides an

environment with cofactors and reductants that would be physiologically relevant for mouse IDO2 activity. The cellular environment permitting significant enzymatic activity of human IDO2 has not yet been well-defined. In addition, testing IDO2 inhibitors in a human population would require genotyping the individuals for the polymorphisms in the *IDO2* gene that are predicted to affect enzymatic activity (Metz et al. 2007). A recent report suggested that response to a combination chloroqiune/radiotherapy treatment for brain metastases was more pronounced in patients with an "active" IDO2 genotype (Eldredge et al. 2012).

A mammalian cell line transfected with mouse IDO2 or mouse IDO1 was used to screen a small library of compounds already approved by the Food and Drug Administration (Bakmiwewa et al. 2012). A number of candidate IDO2 inhibitors were identified, some highly selective for IDO2 inhibition over IDO1. There were several groups of structurally and functionally related compounds identified in this screen. Notably a few classes of drugs contained an imidazole structure, a feature of a previously discovered IDO1 inhibitor, phenyl-imidazole (Sono and Cady 1989). While a drug such as tenatoprazole was highly selective for IDO2 inhibition (IC_{50} 1.8 μM for IDO2 and no inhibition at ≥100 μM for IDO1 or TDO), its other biological effects on gastric secretions would need to be considered in experimental studies (Bakmiwewa et al. 2012).

A striking finding of this study was the number of IDO2-inhibitors identified in the small library. Despite the ratio of substrate to inhibitor being on average 50:1, over 5 % of compounds inhibited IDO2 activity by more than half, through two rounds of screening. In comparison, only one compound inhibited IDO1 to this extent and it was later found to be toxic to both IDO1 and IDO2-expressing cells (Bakmiwewa et al. 2012). This further suggests that IDO2 has different biochemical characteristics to IDO1 and may interact with a wide range of compounds in preference to Trp. An analogy might be to another family of enzymes, the cytochrome monoxygenase P450 enzymes, which interact with a broad range of endogenous and xenobiotic molecules. Although it is not known whether IDO2 metabolizes other substrates, the fact that its Trp-metabolising activity is inhibited by so many compounds, in the presence of excess Trp, suggests that alternative substrates and products should be considered.

Summary and conclusions

The first and rate-limiting step in the kynurenine pathway of Trp metabolism is mediated by three enzymes, TDO, IDO1 and IDO2. The genes for the IDO isoforms are adjacent to each other on mammalian chromosomes and the proteins have a similar secondary and tertiary structure. The two enzymes have evolved to possess distinct expression patterns and biochemical characteristics. Little is still known about the physiological functions of the IDO2 enzyme and the roles it plays in disease conditions involving changes in kynurenine pathway activity, though associations with tumor biology are suspected. It remains to be determined whether there is also some role for IDO2 that may not be related to tryptophan metabolism. The development of siRNA and transgenic/knockout approaches will uncover roles of the enzymes dependent or independent on enzymatic activity. The use of selective inhibitors may provide clues to alternative substrates or interacting molecules. IDO2-selective inhibitors when identified can be used as pharmacological tools to firmly establish its role(s) in the kynurenine pathway and associated disease conditions.

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Conflict of interest None.

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