Amino Acids (2014) 46:2155–2163
DOI 10.1007/s00726-014-1766-3

ORIGINAL ARTICLE

Human indoleamine 2,3-dioxygenase-2 has substrate specificity and inhibition characteristics distinct from those of indoleamine 2,3-dioxygenase-1

Georgios Pantouris · Martynas Serys · Hajime J. Yuasa · Helen J. Ball · Christopher G. Mowat

Received: 7 November 2013 / Accepted: 14 May 2014 / Published online: 30 May 2014

Abstract Indoleamine 2,3-dioxygenase-2 (IDO2) is one of three enzymes (alongside tryptophan 2,3-dioxygenase and indoleamine 2,3-dioxygenase (IDO1)) that catalyse dioxygenation of L-tryptophan as the first step in the kynurenine pathway. Despite the reported expression of IDO2 in tumours, some fundamental characteristics of the enzyme, such as substrate specificity and inhibition selectivity, are still to be clearly defined. In this study, we report the kinetic and inhibition characteristics of recombinant human IDO2. Choosing from a series of likely IDO2 substrates, we screened 54 tryptophan derivatives and tryptophan-like molecules, and characterised the 8 with which the enzyme was most active. Specificity of IDO2 for the two isomers of 1-methyltryptophan was also evaluated and the findings compared with those obtained in other studies on IDO2 and IDO1. Interestingly, IDO2 demonstrates behaviour distinct from that of IDO1 in terms of substrate specificity and affinity, such that we have identified tryptophan derivatives that are mutually exclusive as substrates for IDO1 and IDO2. Our results support the idea that the antitumour activity of 1-Me-D-Trp is unlikely to be related with competitive inhibition of IDO2, and also imply that there are subtle differences in active site structure in the two enzymes that may be exploited in the development of specific inhibitors of these enzymes, a route which may prove important in defining their role(s) in cancer.

Keywords Indoleamine 2,3-dioxygenase-2 · Kynurenine pathway · Substrate · Inhibitor · Specificity

G. Pantouris · M. Serys · C. G. Mowat (✉)
EaStCHEM, School of Chemistry, University of Edinburgh, West Mains Road, Edinburgh EH9 3JJ, UK
e-mail: c.g.mowat@ed.ac.uk

H. J. Yuasa
Laboratory of Biochemistry, Department of Applied Science, Faculty of Science, National University of Science, National University Corporation Kochi University, Kochi 780-8520, Japan

H. J. Ball
Molecular Immunopathology Unit, Discipline of Pathology, School of Medical Sciences, and Bosch Institute, University of Sydney, Sydney, NSW 2006, Australia

Introduction

Indoleamine 2,3-dioxygenase-2 (IDO2) is a third enzyme, along with tryptophan 2,3-dioxygenase (TDO) and indoleamine 2,3-dioxygenase (IDO, or as will be used here to avoid confusion, IDO1), that catalyses the incorporation of molecular oxygen into L-tryptophan in the first step of the kynurenine pathway (Batabyal and Yeh 2007). Human IDO2 (IDO2) was discovered in 2007, and its encoding gene is adjacent to the IDO1 gene on chromosome 8 (Ball et al. 2007; Yuasa et al. 2007; Metz et al. 2007), suggesting that it may have arisen as the product of gene duplication. Nevertheless, the distinct characteristics of IDO2 raise the possibility of the protein having an immunomodulatory role unrelated to that of IDO1. Studies in mice have shown that both IDO1 and IDO2 are expressed in particular tissues (e.g. epididymis), supporting the idea of IDO2 implication in an unidentified signalling pathway (Löb et al. 2009a).

The involvement of IDO2 in immune tolerance and, consequently, its potential importance in tumour escape and cancer was first suggested by Metz et al. (2007), with the presence of IDO2 mRNA being detected in gastric, colon and renal tumours by Löb et al. (2009a). Following this, Sørensen et al. (2011) came to verify the importance of IDO2 in tumour escape and survival by showing that in peripheral blood

samples obtained from both healthy donors and cancer patients there was observed a spontaneous cytotoxic T cell reactivity against IDO2. These T-cells were shown to be cytotoxic effector cells that recognise and kill tumour cells.

The proposed involvement of IDO2 in cancer, in combination with the established immunomodulatory role of IDO, led to these enzymes being potential drug target molecules, and thus the identification and development of specific inhibitors for both enzymes is desirable. Studies on 1-methylated tryptophan derivatives have shown that 1-Me-D-Trp has superior in vivo antitumour activity compared with 1-Me-L-Trp (Hou et al. 2007). For this reason, 1-Me-D-Trp was the subject of clinical trials as an inhibitor of IDO enzymes. Considering the specificity of 1-Me-L-Trp as an inhibitor of IDO (Löb et al. 2009a), it was considered possible that 1-Me-D-Trp antitumour activity could be related to IDO2 function. It has been variously reported that 1-Me-L-Trp is a more effective inhibitor of vertebrate IDO2 (Yuasa et al. 2010; Austin et al. 2010; Qian et al. 2012) than 1-Me-D-Trp, and vice versa (Metz et al. 2007), although the balance of evidence favours 1-Me-L-Trp as the more effective stereoisomer.

The different reported behaviours of the stereoisomers of 1-Me-Trp, in terms of inhibition of purified enzymes and their effects on various cell types, have been suggested to be related to differential transport into cells, or compartmentalization therein. In addition it is possible that this may be related to variations in enzymatic metabolism or inhibition of IDO1 via mechanisms other than conventional competitive inhibition. The fact that there exists both IDO1 and IDO2 further complicates attempts to explain these effects (Löb et al. 2009b). The recent identification of selective IDO2 inhibitors (Bakmiwewa et al. 2012) will be useful in addressing these uncertainties and helping to define the distinct biological roles of the tryptophan and indoleamine 2,3-dioxygenases.

In the present study, we describe the characterisation of recombinant human IDO2 (IDO2) with respect to its substrate specificity and inhibition selectivity. These data are compared with the corresponding data for IDO, and potential similarities and differences were evaluated. This work, in combination with previous findings, suggests that the physiological function of IDO2 may be distinct from that of IDO1, while illustrating subtle differences in substrate specificity and binding affinities that may help inform the development of specific inhibitors of these enzymes.

Materials and methods

Materials

Most of the chemicals used (L-Trp, D-Trp, L-ascorbate, bovine liver catalase, methylene blue and tryptophan analogues) including those for buffers (Sigma-Aldrich) were of the highest analytical grade (≥97 % purity) and were used without further purification. 1-Me-L-Trp and 1-Me-D-Trp (95 % purity, Sigma-Aldrich) were purified further as described previously (Chauhan et al. 2009).

Cloning and expression of IDO1 and IDO2

The full-length human IDO1 construct used was as described by Papadopoulou et al. (2005), while the human IDO2 construct encoded an N-terminal truncated version of the predicted protein sequence consisting of residues 14–420 (as suggested by Ball et al. 2007). In order to prepare this construct, single-stranded cDNA was synthesized from human liver RNA (Clontech, cat. #636531) using the First-Strand cDNA Synthesis Kit (GE Healthcare) and oligo-dT primer. The human IDO2 cDNA was amplified by PCR using the KOD plus DNA polymerase (Toyobo). Specific primers used for PCR were: 5′-CACCATGGAGCCCCACAGACCG-3′ (contained a CACC overhang at the 5′ end for TOPO cloning) and 5′-CTAACCACGTGGGTGAAG-3′. The product was inserted directionally into pENTR™/D-TOPO® vector (Life Technologies). The inserts were then transferred to the pDEST™17 vector via LR recombination reaction (Life Technologies). The nucleotide sequences of the construct were confirmed by sequencing.

Cultures of *Escherichia coli* BL21 (DE3) containing the IDO1 and IDO2 encoding plasmids were grown in Luria–Bertani (LB) broth previously sterilised by autoclaving at 121 °C for 20 min. Starter cultures for large-scale growth were prepared by inoculating 20 ml of sterile media (125 μg ml^{-1} in ampicillin) with IDO1 and IDO2 dimethyl sulfoxide cell stocks via sterile pipette tips, and incubated with shaking for 24 h at 37 °C, 225 rpm. Growth flasks containing 600 ml of LB broth were supplemented with 20 μg ml^{-1} of ampicillin and inoculated with 1 ml of IDO1 and IDO2 starter cultures. Flasks were incubated with shaking at 37 °C and 200 rpm until the OD_{600} reached 0.6. Protein production was then induced by addition of isopropyl-β-D-1-thiogalactopyranoside to a final concentration of 250 mg l^{-1}. The culture media were supplemented with 0.5 ml of 5 mM hemin solution per flask to maximise heme incorporation, and the temperature decreased to 22 °C. Flasks were left for a further 12 h with shaking at 135 rpm until the cells were harvested by centrifugation (10 min at 11,000×*g*) using a Sorvall RC-5B refrigerated centrifuge at 4 °C.

Protein purification and handling

Harvested cells containing IDO1 or IDO2 were resuspended in 20 mM TrisHCl buffer pH 8.0, 300 mM in

NaCl, 10 mM in imidazole, 1 mM in *tris*(2-carboxyethyl)phosphine (TCEP). Cell lysis was initiated by incubating 200 ml of cell suspension for 30 min with hen egg white lysozyme (1 mg per ml of suspension), phenylmethylsulfonyl fluoride (2 mg per ml of suspension) and 1 complete protease inhibition tablet (Sigma). Lysis was completed by ultrasonication of the suspension on ice. Cell debris was then removed via centrifugation for 1 h at 28,000×*g* and at 4 °C, and the supernatant collected. The supernatant was then loaded onto a Ni-agarose column and the bound material washed with 5 column volumes (CV) of 20 mM Tris–HCl pH 8.0, 300 mM in NaCl, 20 mM in imidazole, 1 mM in TCEP. Following this step, IDO1 or IDO2 was eluted using the same buffer but with a higher concentration of imidazole (250 mM). Size exclusion chromatography (Superdex 75) was also used in order to remove imidazole. The column was pre-incubated in 20 mM Tris–HCl pH 8.0, 1 mM in TCEP for at least 1 CV and the protein collected judged to be pure and homogeneous by SDS-PAGE. The level of heme incorporation in pure recombinant IDO1 and IDO2 was ascertained based on the ratio of the absorbances at 405 nm (the oxidised heme Soret peak) and 278 nm (general aromatic amino acid absorbance) in the UV/visible spectra of the enzymes. In the case of IDO1, the A_{405}/A_{278} ratio was 1.5 and in the case of IDO2 it was 1.7, indicating >95 % heme incorporation in both enzymes.

Determination of pH dependence

Determination of the optimum pH for IDO2 activity was carried out by monitoring the rate of formation of N-formyl-L-kynurenine in 100 mM potassium phosphate (KP_i) buffer with pH values between 5.8 and 8.0. The rate of formation of N-formyl-L-kynurenine was calculated using the change in absorbance at 321 nm, the wavelength at which the product has maximum absorbance. The final concentrations of the assay components were 20 mM ascorbate, 10 μM methylene blue and 10 μg ml^{-1} catalase. The enzyme and L-Trp concentrations were kept constant at 1.8 μM and 4 mM, respectively, and the formation of *N*-formyl-L-kynurenine was monitored at increments of 0.2 pH units. The experiment was carried out in a cuvette with a 1-cm path length, and the change in absorbance was measured using a Shimadzu UV-2101PC UV/vis spectrophotometer with the temperature kept at 25 °C using a thermostatically controlled water bath. Data obtained were analyzed using Microcal Origin software.

Enzyme kinetics and substrate screening

Initial screening of the 54 candidate IDO2 substrate molecules was carried out in a 96-well microplate with the components dissolved in 100 mM KP_i pH 7.5. Each well contained 200 μl of assay mixture, containing 400 mM ascorbic acid, 1 mM methylene blue, 10 mg ml^{-1} catalase, the candidate molecule at 1 mM final concentration and 4 μM IDO2. The reaction mixture was incubated at room temperature for 30 min and the reaction terminated by addition of 40 μl of trichloroacetic acid (30 % w/v) into each well. Subsequently the microplate was transferred into an oven and incubated at 50 °C for 30 min. The microplate was then centrifuged for 15 min at 4,000 rpm and 125 μl of the supernatant transferred to a new microplate and mixed with equal volume of 4-dimethylaminobenzaldehyde (DMAB) in acetic acid (2 % w/v). Finally, the absorbance was measured at 490 nm, where the kynurenine-DMAB complex has a maximum. Any reaction of the substrate with DMAB in the absence of IDO2 was controlled for.

Kinetic analyses of IDO1 and IDO2 were carried out using a method adapted from Basran et al. (2008) and Chauhan et al. (2009) by observing the increase in absorbance at 321 nm over time as *N*-formylkynurenine ($\varepsilon_{321} = 3{,}750\ M^{-1}\ cm^{-1}$) or its analogues were formed. Each experiment was carried out either in 100 mM KP_i pH 6.5 (IDO1) or 100 mM KP_i pH 7.5 (IDO2). All the assay components were dissolved in the appropriate buffer at final concentrations of 20 mM ascorbate, 10 μM methylene blue and 10 μg ml^{-1} catalase. The concentration of IDO1 was varied between 0.4 and 3.4 μM, with substrate concentrations ≤4 mM. In the case of IDO2, the enzyme concentration was varied between 0.4 and 2 μM while substrate concentrations ≤40 mM were used. Assays were carried out under aerobic conditions at 25 °C using a thermostatically controlled water bath. Data obtained were fitted to the Michaelis–Menten equation and analyzed using Microcal Origin software. The formation of *N*-formylkynurenine (from L-tryptophan) and the analogous product resulting from dioxygenation of 5-Me-D,L-Trp by IDO2 was confirmed by ESI–MS analysis of the reaction mixture. It was therefore assumed that *N*-formylkynurenine analogues formed from tryptophan analogues were as anticipated.

IDO1 and IDO2 inhibition assays

The IDO1 and IDO2 inhibition assays were carried out according to the method of Takikawa et al. (1988) with minor modifications. The assays were carried out in 96-well microplates with the components dissolved in either 100 mM KP_i pH 6.5 or 100 mM KP_i pH 7.5, for IDO1 and IDO2, respectively. Each well contained 200 μl of assay mixture. The reaction mixture was comprised 400 mM ascorbic acid, 1 mM methylene blue, 10 $mgml^{-1}$ catalase, L-tryptophan at final assay concentrations between

≤45 μM (IDO1) and ≤8 mM (IDO2), inhibitor and the enzyme at a final concentration of 15 nM (IDO1) and 900 nM (IDO2). The reaction mixture was incubated at room temperature for either 15 min (IDO1) or 30 min (IDO2) and the reaction subsequently terminated by adding 40 μl of trichloroacetic acid (30 % w/v) into each well. Subsequently the microplate was transferred into an oven and incubated at 50 °C for 30 min. The microplate was then centrifuged for 15 min at 4,000 rpm and 125 μl of the supernatant transferred to a new microplate and mixed with equal volume of DMAB in acetic acid (2 % w/v). Finally, the absorbance was measured at 490 nm, where the kynurenine-DMAB complex has a maximum. Due to the fact that some inhibitor molecules are also 'slow' substrates for IDO2, any background inhibitor turnover (as measured by the formation of a DMAB adduct in the absence of substrate) was taken into account as a control measure in determining K_i for the inhibitors studied.

Results and discussion

Determination of the optimum pH and buffer conditions

Prior to the biochemical characterization of IDO2, it was necessary to ascertain the optimum pH for function of the enzyme. For this reason, the activity of IDO2 was measured in 100 mM potassium phosphate buffer at pH values between pH 5.8 and 8.0. The findings clearly showed that optimal enzymatic activity occurs around pH 7.5. This finding is in good agreement with previously reported data for recombinant mouse IDO2, where the optimal pH for activity was also found to be 7.5 (Austin et al. 2010). This is perhaps unsurprising because the human and mouse enzymes share 74 % sequence identity (Chang et al. 2009). Examination of several buffer compositions at pH 7.5 indicated 100 mM potassium phosphate buffer as the most suitable for this study.

Kinetic analysis

The kinetic parameters (K_m, k_{cat} and k_{cat}/K_m) obtained for IDO2 activity with L-Trp, D-Trp and a number of tryptophan analogues are shown in Table 1. The structures of the characterised substrates are also shown in Fig. 1 and Michaelis–Menten plots are shown in Fig. 2. These molecules were chosen from the 54 potential substrates that were screened, as the most effective substrates for IDO2 as measured by greater turnover than was observed with L-Trp, D-Trp and their 1-methylated derivatives in the initial screen. From Table 1, it can be seen that IDO2 shows greatest catalytic efficiency with 5-methoxytryptophan, largely due to its low K_m value (547 ± 29 μM).

Table 1 Kinetic parameters for IDO2 with L-Trp and other tryptophan analogues

Name	k_{cat}/K_m ($M^{-1}s^{-1}$)	K_m (μM)	k_{cat} (s^{-1})
5-MeO-D,L-Trp	294	547 ± 29	0.161 ± 0.003
5-Me-D,L-Trp	229	1,570 ± 188	0.36 ± 0.02
6-Me-D,L-Trp	31	3,457 ± 962	0.108 ± 0.013
5-F-D,L-Trp	28	1,768 ± 161	0.049 ± 0.002
1-Me-L-Trp	16	696 ± 41	0.011 ± 0.006
L-Trp	15	6,809 ± 917	0.103 ± 0.006
1-Me-D-Trp	7.0	747 ± 168	0.0052 ± 0.0004
D-Trp	2.8	3,609 ± 675	0.0103 ± 0.0006

The molecules are in order of catalytic efficiency, based on k_{cat}/K_m values. Substrates such as 5-OH-L-Trp and melatonin revealed negligible activity and for that reason are not included in the table. Michaelis-Menten plots from which these data were obtained are shown in Fig. 2. All figures quoted are the mean ± SEM of 3 replicate measurements

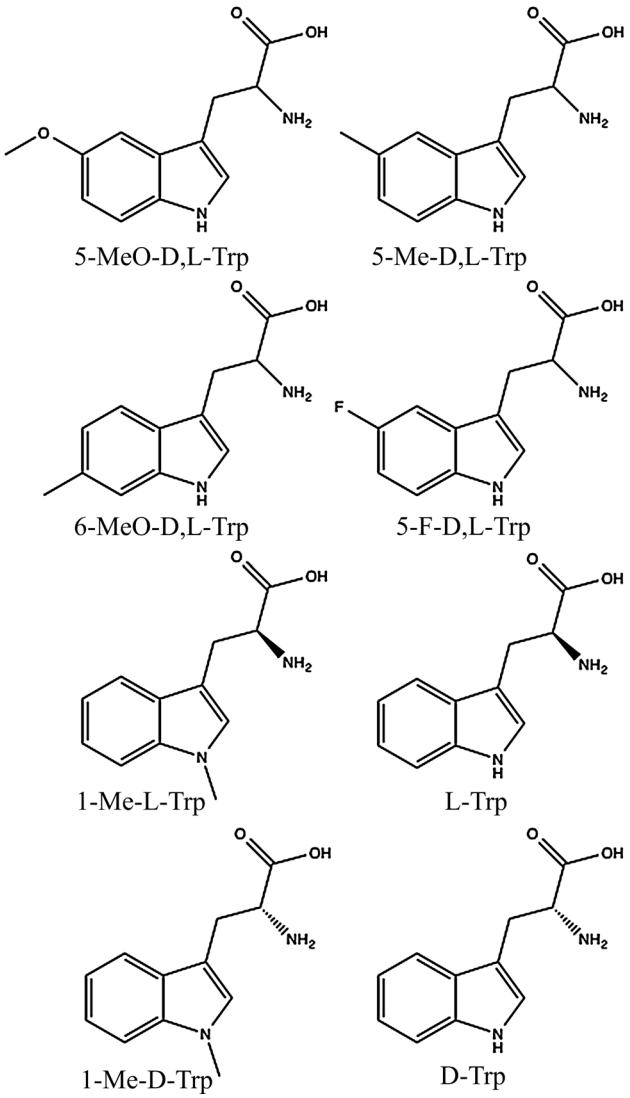

Fig. 1 The structures of tryptophan derivatives that were used in this work

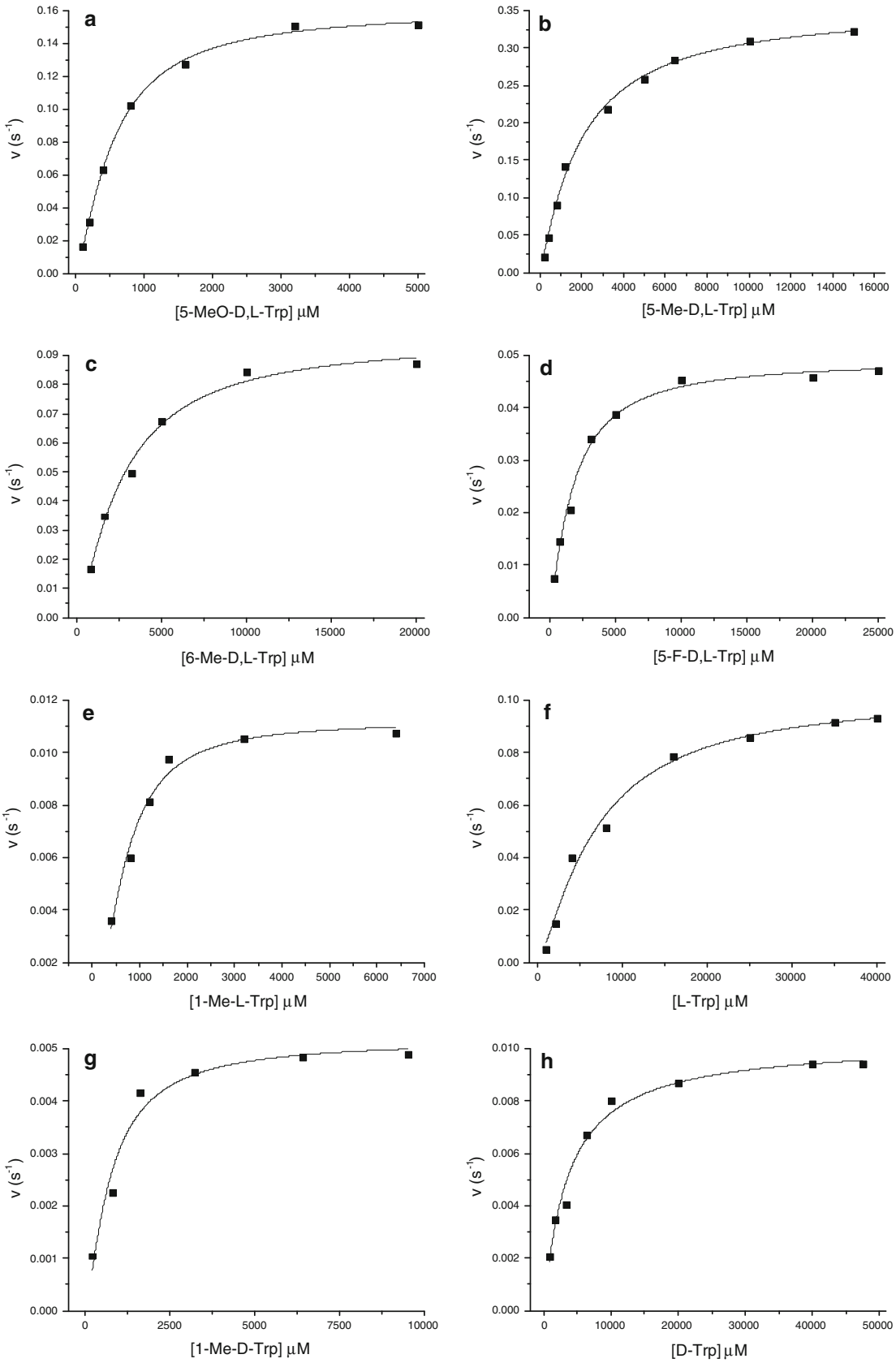

Fig. 2 Michaelis–Menten plots for the eight IDO2 substrates shown in Fig. 1. All data points are the mean of three independent measurements carried out at 25 °C in 100 mM potassium phosphate buffer, pH 7.5

Interestingly however, replacement of the 5-methoxy group by a methyl group has a negative influence in the binding of the molecule, decreasing its affinity threefold. On the other hand, this modification positively affects the turnover rate, increasing k_{cat} from 0.161 ± 0.003 s^{-1} for 5-methoxytryptophan to 0.36 ± 0.02 s^{-1} for 5-methyltryptophan, perhaps due to the differing electron withdrawing/electron donating characters of the 5-substituents. Furthermore, moving the methyl group to the 6- position has the effect of decreasing both the affinity of the enzyme for the molecule and its turnover rate, resulting in a decrease in k_{cat}/K_m from 229 $M^{-1}s^{-1}$ for 5-methyltryptophan to 31 M^{-1} s^{-1} for 6-methyltryptophan (Table 1). In the case of 5-fluorotryptophan, it seems that the electron-withdrawing properties of fluorine influence both affinity and turnover rate in a negative manner. Despite this, 5-fluorotryptophan is a better substrate for IDO2 than the presumed natural substrate of the enzyme, L-Trp. 1-Me-L-Trp and L-Trp displayed similar overall efficiencies as substrates but dissimilar kinetic parameters. Binding of 1-Me-L-Trp is more favourable than binding of L-Trp (which has the highest K_m value of all substrates tested), although L-Trp turnover occurs around 10 times faster than for 1-Me-L-Trp. In comparison with 1-Me-L-Trp, the D-enantiomer of 1-Me-Trp showed a similar binding affinity (K_m = 747 ± 168 μM) but lower turnover rate (k_{cat} = 0.0052 ± 0.0004 s^{-1}). Interestingly, the 1-Me-Trp stereoisomers display the lowest K_m values of all substrates mentioned here, with only these and the 5-MeO-D,L-Trp racemic mixture giving sub-millimolar values. Previous in vitro kinetic work on human IDO2 using fluorescence-based assays was unable to resolve K_m and k_{cat} values due to the inability to achieve saturation with L-Trp at concentrations up to 4 mM, in line with the >6 mM K_m value observed here (Meininger et al. 2011). Other studies on the recombinant mouse IDO2 have indicated L-Trp K_m values of the same order of magnitude as that presented here for the human enzyme (Austin et al. 2010).

Inhibition of activity

Due to the discovery of IDO2 mRNAs in a variety of human cancers (Löb et al. 2009a), and the strong inhibition effect of 1-Me-D-Trp in mouse tumour models (Hou et al. 2007), it is desirable to clarify the selectivity of 1-methyltryptophan inhibition of IDO1 and IDO2. Consequently, the effectiveness of inhibition was investigated with D-Trp, 1-Me-L-Trp and 1-Me-D-Trp as inhibitors of recombinant human IDO2, and the findings are illustrated in Fig. 3. The fairly high affinities (low K_m) of both 1-Me-L-Trp and 1-Me-D-Trp for the enzyme, (K_m = 696 ± 41 μM and

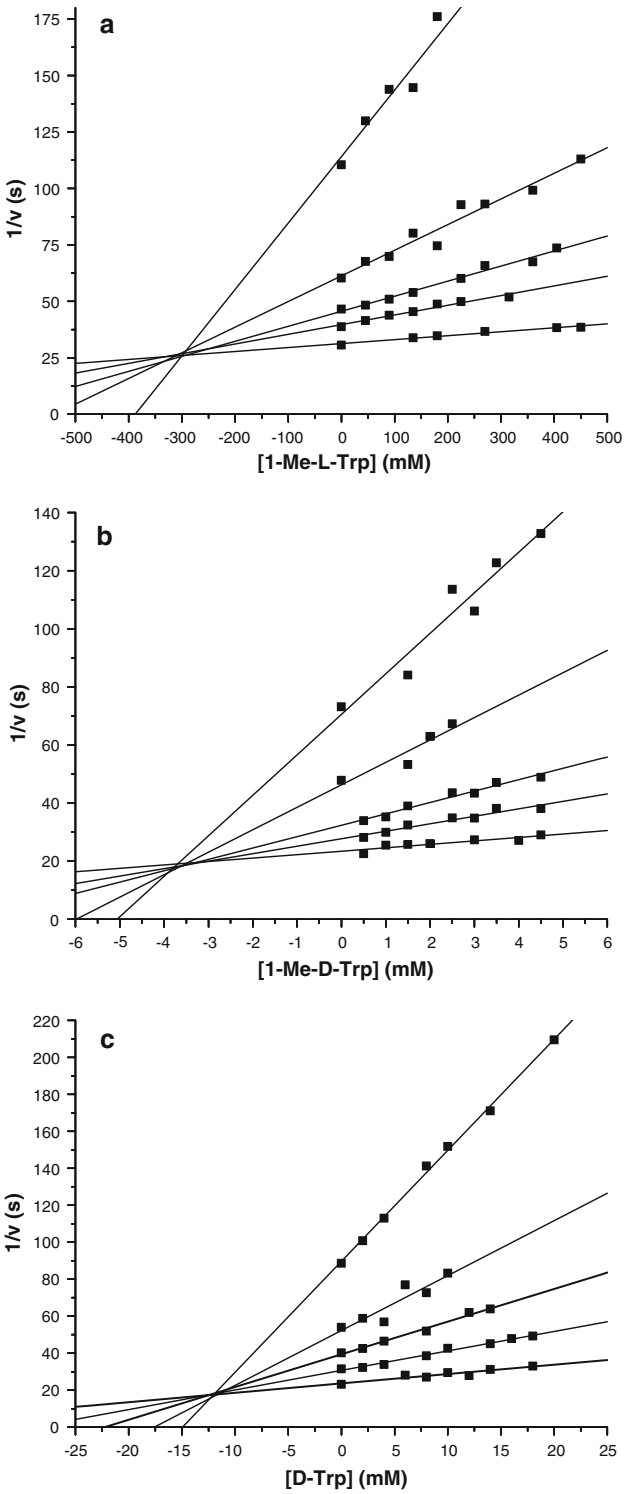

Fig. 3 Inhibition of IDO2 using **a** 1-Me-L-Trp, **b** 1-Me-D-Trp and **c** D-Trp as inhibitors. The experiment was carried out in 100 mM KPi buffer pH 7.5 with the enzyme concentration kept constant at 900 nM. In the case of 1-Me-L-Trp and 1-Me-D-Trp the substrate concentrations used were 1, 2, 3, 4 and 6 mM, while for D-Trp the substrate concentrations were 1, 2, 3, 5 and 7 mM. Individual data points represent the mean of three independent measurements

$K_m = 747 \pm 168$ μM, respectively) in combination with their poor turnover rates, enable inhibition of IDO2 by both molecules. In contrast with these methylated derivatives, D-Trp showed weak binding affinity and is unlikely to represent an effective inhibitor of the enzyme. These findings are summarised in Table 2. For comparison, data for IDO1 are also given. These results clearly show that of these molecules 1-Me-L-Trp is the best inhibitor of recombinant human IDO2. In comparison with the D-enantiomer, 1-Me-L-Trp is ~ tenfold more potent as an inhibitor. Considering that 1-Me-D-Trp is not found to inhibit IDO1 (or indeed mouse IDO2), the albeit weak inhibition ability of the molecule with human IDO2 should be mentioned. In line with the turnover data, D-Trp is an implausible inhibitor of IDO2 with an inhibition constant of 12 mM. The IC_{50} value for inhibition of IDO2 by 1-Me-L-Trp has been determined using a fluorescence-based assay and has been determined to be 35 μM (Yuasa et al. 2010), while other determinations of K_i values have been carried out using the recombinant mouse IDO2 (Austin et al. 2010). In this case, it was found that 1-Me-L-Trp inhibited L-Trp turnover with a K_i of 2,750 μM (under similar assay conditions to those used here).

In comparison with IDO1, it is clear that IDO2 demonstrates differing behaviour with L-Trp and its analogues. Indeed, in the case of IDO1, L-Trp substrate inhibition is observed (Efimov et al. 2012). This phenomenon is not observed for IDO2 and this is in agreement with previous observations on IDO2 from other species (Yuasa et al. 2010). The fact that 1-Me-L-Trp (rather than 1-Me-D-Trp) is the better inhibitor of IDO2 (and IDO1) raises questions about the origin of the anti-tumour activity displayed by 1-Me-D-Trp, with the suggestion that 1-Me-D-Trp can act as a tryptophan mimetic, thus preventing autophagy, being one possible explanation (Metz et al. 2012).

Table 2 Inhibition of IDO1 and IDO2 by D-Trp, 1-Me-L-Trp and 1-Me-D-Trp. Figures quoted are the mean ± SEM of three replicate measurements

	L-Trp K_{is} (μM)	D-Trp K_i (μM)	1-Me-L-Trp K_i (μM)	1-Me-D-Trp K_i (μM)
IDO1	50[19]	543 ± 35	18.0 ± 3.4	ni
IDO2	ni	12,000 ± 2000	300 ± 25	3300 ± 400

Comparison of IDO1 with IDO2

The study of IDO2 substrate and inhibition specificity shows that the enzyme is very inefficient as an L-Trp dioxygenase, the physiological function of IDO1. Regarding the kinetic parameters (K_m, k_{cat}) and substrate specificity of the two enzymes, important dissimilarities were observed. Data for IDO and IDO2 are summarised and presented in Table 3. Molecules like 5-MeO-D,L-Trp and 6-Me-D,L–Trp are shown to be substrates for IDO2 (Table 1), something which has not been found for IDO1. In this study, we also examined the potency of 5-HO-L-Trp and melatonin as substrates for IDO2. While neither of them showed any activity for IDO2, 5-HO-L-Trp is a published substrate of IDO with good affinity for the enzyme ($K_m = 17.0 \pm 1.1$ μM) but poor turnover rate ($k_{cat} = 0.0250 \pm 0.0004$ s^{-1}) (Basran et al. 2008). Interestingly, 5-Me-D,L-Trp was found to be, relatively speaking, a very good substrate for IDO1 and a very poor substrate for IDO2. Similarly, 5-F-D,L-Trp demonstrated different behaviours for IDO1 and IDO2. In the case of IDO1, 5-F-D,L-Trp is a good substrate with activity similar to that found for L-Trp (Table 3). In addition, 5-F-D,L-Trp showed high affinity for the enzyme ($K_m = 6 \pm 1$ μM), higher even than that for L-Trp ($K_m = 20.90 \pm 3.95$ μM). For IDO2, though the findings differ, with the 5-fluoro substrate showing lower activity than 5-Me-D,L-Trp. Examination of L-Trp turnover revealed surprising kinetic parameters for IDO2. According to the various enzymatic efficiencies presented in Table 1, L-Trp is the sixth most active substrate for IDO2, with a level of activity that seems unlikely to be biologically relevant. Amongst the several molecules that have been characterised as substrates for IDO2, the L-Trp data revealed the weakest binding affinity (K_m ~ 6.8 mM). While both 1-Me-L-Trp and 1-Me-D-Trp are inhibitors of IDO2 it is apparent that, like in IDO1, 1-Me-L-Trp is the better of the pair.

Table 3 Kinetic characteristics for IDO1 and IDO2 using L-Trp and other tryptophan analogues as substrates

Assays of IDO1 and IDO2 were carried out at 25 °C in 100 mM KP_i pH 6.5 and 7.5, respectively. Figures quoted are the mean ± SEM of three replicate measurements.

[a] Values taken from Basran et al. (2008)

Substrate	IDO1		IDO2	
	k_{cat} (s^{-1})	K_m (μM)	k_{cat} (s^{-1})	K_m (μM)
L-Trp	2.97 ± 0.20	20.90 ± 3.95	0.103 ± 0.006	6809 ± 917
D-Trp	2.7 ± 0.3	296 ± 19	0.0103 ± 0.0006	3,609 ± 675
1-Me-L-Trp	0.062 ± 0.001	70 ± 1	0.011 ± 0.006	696 ± 41
1-Me-D-Trp	0.095 ± 0.007	660 ± 43	0.0052 ± 0.0004	747 ± 168
5-F-D,L-Trp	0.76 ± 0.01[a]	6 ± 1	0.049 ± 0.002	1,768 ± 161
5-Me-D,L-Trp	3.78 ± 0.16[a]	98 ± 14	0.36 ± 0.02	1570 ± 188
5-HO-L-Trp	0.0250 ± 0.0004[a]	17 ± 1	No activity	–

What is evident from the data presented here is that IDO2 is considerably less efficient as a dioxygenase than IDO1, as evidenced by a combination of higher binding constants for its substrates and very low turnover rates. In addition, there are some curious anomalies between the activities of the two enzymes with structurally similar substrates—5-OH-Trp and 5-MeO-Trp are found to be exclusively substrates for IDO1 and IDO2, respectively, for example. These findings suggest that there may be potentially important differences in substrate binding/active site structure between the two enzymes, thus potentially increasing the possibility of identifying and/or developing IDO2-specific inhibitors. Taking all of these data together, it is apparent that IDO2 is a 'slow' and inefficient enzyme in comparison with IDO1, at least under the conditions used here. This has been recognised in prior work, with the methylene blue/ascorbate-based assay leading to measurement of generally lower enzymatic rates and lower affinities for L-Trp than are observed using a cytochrome b_5-based assay (Austin et al. 2010; Meininger et al. 2011).

Indeed, it has been suggested that in humans, IDO2 may have evolved to be biologically inactive, may have a role independent of enzymatic activity, or that the conditions under which it is most active have not yet been fully established. This idea that IDO2 may be activated under specific conditions is intriguing, and the finding by Lo et al. (2011) that culture of human basal carcinoma cells with the chemokine CXCL11 induced kynurenine production that was correlated with expression of *IDO2* mRNA (but not *IDO1* mRNA) would suggest that IDO2 is responsible for the kynurenine produced. Thus, the possibility exists that IDO2 is activated in certain cell types and/or under certain specific conditions.

Conclusion

The aim of this study was to examine the biochemical behaviour of recombinant human IDO2 and from there to extract useful conclusions about its function and catalytic preferences. After the discovery of IDO2 and its characterization as a third enzyme that catalyses incorporation of dioxygen into L-tryptophan, it had been proposed that IDO2 is likely to be an evolutionary ancestor of IDO1, mainly because of its moderate ability to produce N-formylkynurenine. Nevertheless, the distinct expression pattern and response to extracellular stimuli reinforced the idea that IDO2 is a non-redundant enzyme with a distinct physiological role (Chang et al. 2009). This work summarises the behaviour of IDO2 with respect to turnover of, and inhibition by, various tryptophan derivatives. Although, in comparison with IDO1, IDO2 appears to be a considerably less efficient enzyme, there are some potentially important distinctions that may be exploited in the development of specific inhibitors. Our findings support the idea of a non-redundant enzyme with a distinct biological role.

Conflict of interest The authors declare that they have no conflict of interest.

References

Austin CJD, Mailu BM, Maghzal GJ, Sanchez-Perez A, Rahlfs S, Zocher K, Yuasa HJ, Arthur JW, Becker K, Stocker R et al (2010) Biochemical characteristics and inhibitor selectivity of mouse indoleamine 2,3-dioxygenase-2. Amino Acids 39:565–578

Bakmiwewa SM, Fatokun AA, Tran A, Payne RJ, Hunt NH, Ball HJ (2012) Identification of selective inhibitors of indoleamine 2,3-dioxygenase. Bioorg Med Chem Lett 22:7641–7646

Ball HJ, Sanchez-Perez A, Weiser S, Austin CJD, Astelbauer F, Miu J, McQuillan JA, Stocker R, Jermiin LS, Hunt NH (2007) Characterization of an indoleamine 2,3-dioxygenase-like protein found in humans and mice. Gene 396:203–213

Basran J, Rafice SA, Chauhan N, Efimov I, Cheesman MR, Ghamsari L, Raven EL (2008) A kinetic, spectroscopic, and redox study of human tryptophan 2,3-dioxygenase. Biochemistry 47:4752–4760

Batabyal D, Yeh SR (2007) Human tryptophan dioxygenase: a comparison to indoleamine 2,3-dioxygenase. J Am Chem Soc 129:15690–15701

Chang MY, Metz R, Muller AJ, Prendergast GC (2009) IDO2 (indoleamine 2,3-dioxygenase 2) Atlas Genet Cytogenet Oncol Haematol. http://AtlasGeneticsOncology.org/Genes/IDO2ID44387ch8p11.html

Chauhan N, Thackray SJ, Rafice SA, Eaton G, Lee M, Efimov I, Basran J, Jenkins PR, Mowat CG, Chapman SK, Raven EL (2009) Reassessment of the reaction mechanism in the heme dioxygenases. J Am Chem Soc 131:4186–4187

Efimov I, Basran J, Sun X, Chauhan N, Chapman SK, Mowat CG, Raven EL (2012) The mechanism of substrate inhibition in human indoleamine 2,3-dioxygenase. J Am Chem Soc 134:3034–3041

Hou DY, Muller AJ, Sharma MD, DuHadaway J, Banerjee T, Johnson M, Mellor AL, Prendergast GC, Munn DH et al (2007) Inhibition of indoleamine 2,3-dioxygenase in dendritic cells by stereoisomers of 1-methyl-tryptophan correlates with antitumor responses. Cancer Res 67:792–801

Lo BK, Jalili RB, Zloty D, Ghahary A, Cowan B, Dutz JP, Carr N, Shapiro J, McElwee KJ (2011) CXCR3 ligands promote expression of functional indoleamine 2,3-dioxygenase in basal cell carcinoma keratinocytes. Br J Dermatol 165:1030–1036

Löb S, Königsrainer A, Zieker D, Brücher BLDM, Rammensee HG, Opelz G, Terness P (2009a) IDO1 and IDO2 are expressed in human tumors: Levo- but not dextro-1-methyl tryptophan inhibits tryptophan catabolism. Cancer Immunol Immunother 58:153–157

Löb S, Königsrainer A, Rammensee HG, Opelz G, Terness P (2009b) Inhibitors of indoleamine-2,3-dioxygenase for cancer therapy: can we see the wood for the trees? Nat Rev Cancer 9:445–452

Meininger D, Zalameda L, Liu Y, Stepan LP, Borges L, McCarter JD, Sutherland CL (2011) Purification and kinetic characterization of human indoleamine 2,3-dioxygenases 1 and 2 (IDO1 and IDO2) and discovery of selective IDO1 inhibitors. Biochim Biophys Acta 1814:1947–1954

Metz R, DuHadaway JB, Kamasani U, Laury-Kleintop L, Muller AJ, Prendergast GC et al (2007) Novel tryptophan catabolic enzyme IDO2 is the preferred biochemical target of the antitumor indoleamine 2,3-dioxygenase inhibitory compound D-1-methyl-tryptophan. Cancer Res 67:7082–7087

Metz R, Rust S, Duhadaway JB, Mautino MR, Munn DH, Vahanian NN, Link CJ, Prendergast GC (2012) IDO inhibits a tryptophan sufficiency signal that stimulates mTOR: a novel IDO effector pathway targeted by D-1-methyltryptophan. Oncoimmunology 1:1460–1468

Papadopoulou ND, Mewies M, McLean KJ, Seward HE, Svistunenko DA, Munro AW, Raven EL (2005) Redox and spectroscopic properties of human indoleamine 2,3-dioxygenase and a His303Ala variant: implications for catalysis. Biochemistry 44:14318–14328

Qian F, Liao J, Villella J, Edwards R, Kalinski P, Lele S, Shrikant P, Odunsi K (2012) Effects of 1-methyltryptophan stereoisomers on IDO2 enzyme activity and IDO2-mediated arrest of human T cell proliferation. Cancer Immunol Immunother 61:2013–2020

Sørensen RB, Køllgaard T, Andersen RS, van den Berg JH, Svane IM, Straten PT, Andersen MH et al (2011) Spontaneous cytotoxic T-cell reactivity against indoleamine 2,3-dioxygenase-2. Cancer Res 71:2038–2044

Takikawa O, Kuroiwa T, Yamazaki F, Kido R (1988) Characterization of indoleamine 2,3-dioxygenase in various human cells induced by IFN-γ and evaluation of the enzyme-mediated tryptophan degradation in its anticellular activity. J Biol Chem 263:2041–2048

Yuasa HJ, Takubo M, Takahashi A, Hasegawa T, Noma H, Suzuki T (2007) Evolution of vertebrate indoleamine 2,3-dioxygenases. J Mol Evol 65:705–714

Yuasa HJ, Ball HJ, Austin CJD, Hunt NH (2010) 1-L-Methyltryptophan is a more effective inhibitor of vertebrate IDO2 enzymes than 1-D-methyltryptophan. Comp Biochem Physiol B 157:10–15