Research Article

Future
Medicinal Chemistry

Binding properties of different categories of IDO1 inhibitors: a microscale thermophoresis study

Aim: Inhibition of IDO1 is a strategy pursued in the immune-oncology pipeline for the development of novel anticancer therapies. At odds with an ever-increasing number of inhibitors being disclosed in the literature and patent applications, only very few compounds have hitherto advanced in clinical settings. **Materials & methods:** We have used MicroScale Thermophoresis analysis and docking calculations to assess on a quantitative basis the binding properties of distinct categories of inhibitors to IDO1. **Results:** Results shed further light on hidden molecular aspects governing the recognition by the enzyme of compounds with different mechanism of inhibition. **Conclusion:** Results pinpoint specific binding features of distinct inhibitors to IDO1 that offer clues for the design of next-generation inhibitors of the enzyme.

Francesco Antonio Greco[1], Alice Coletti[1], Chiara Custodi[1], Daniela Dolciami[1], Alessandro Di Michele[2], Andrea Carotti[1], Maura Marinozzi[1], Nina Schlinck[3] & Antonio Macchiarulo*,[1]

[1]Dipartimento di Scienze Farmaceutiche, University of Perugia, Via del Liceo 1, 06123 Perugia, Italy
[2]Dipartimento di Fisica e Geologia, University of Perugia, Via Pascoli, 06123 Perugia, Italy
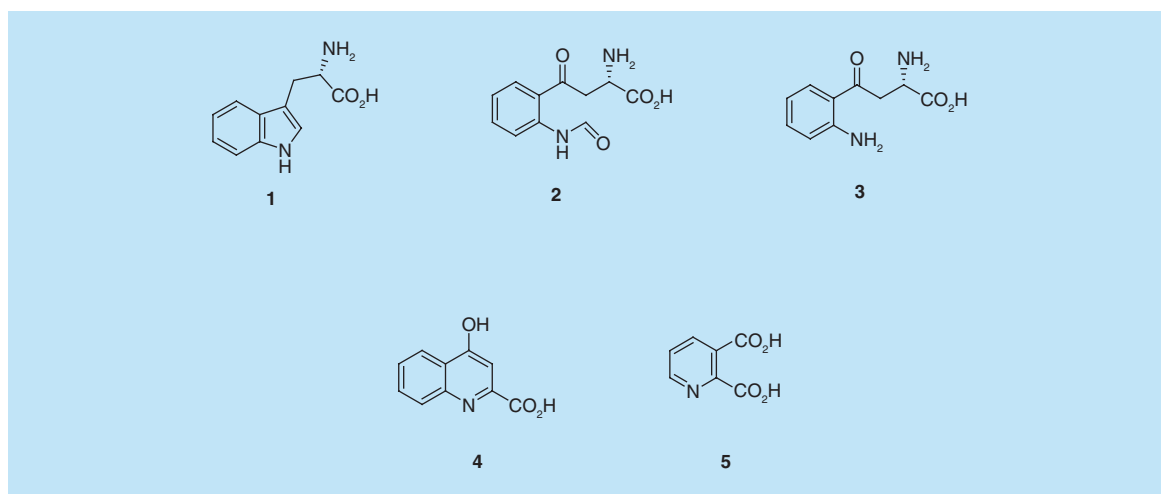[3]NanoTemper Technologies GmbH, Floessergasse 4, 81369 München, Germany
*Author for correspondence:
Tel.: +39 075 585 5160
Fax: +39 075 585 5161
antonio.macchiarulo@unipg.it

In the last 40 years, *L*-Tryptophan (*L*-Trp) catabolism has gained attention because of its importance in immunity [1,2]. The 95% of dietary *L*-Trp (**1**, Figure 1) is catabolized through the kynurenine pathway (KP), with IDO1 being a heme-containing enzyme that catalyzes the first and rate-limiting step of the pathway to produce *N*-formyl-kynurenine (**2**) [3]. Many downstream products of KP have been identified as biologically active compounds (**3–5**), showing the capability to regulate inflammatory responses through several mechanisms [4]. An increasing body of evidence has thus thrust IDO1 under the spotlight as a key druggable target to control the balance between immune-tolerance and immune-inflammatory responses [5,6]. Noteworthy, signaling functions of IDO1 have also been discovered [7,8]. Specifically, phosphorylation on immune-receptor tyrosine-based inhibition motifs (ITIMs) of IDO1 bestows specific properties to the enzyme enabling its interaction with protein partners

such as SHP-1 and -2 or SOCS-3 [9,10]. Although the former case leads to prolongation of IDO1's half-life and long-term immune-tolerance; in the latter case, IDO1 is tagged for proteasome degradation eventually leading to inflammatory response. These autoregulatory signaling functions add on the effects arising from the catalytic activity of the enzyme that are mediated by consumption of *L*-Trp (**1**) and/or production of bioactive KP metabolites (**3–5**) [11–13].

The breakthrough discovery that IDO1 plays a pivotal role in cancer immune-editing process has fostered an intense research activity aimed at developing inhibitors of the enzyme as novel anticancer agents [14–16]. IDO1 is indeed overexpressed in many human cancer cells as well as in dendritic cells presenting tumor antigens [17]. Cancer cells exploit IDO1 functions to propel their growth, creating an immune-privileged tolerogenic microenvironment in which cytotoxic T cells are starved by *L*-Trp

newlands press part of fsg

Figure 1. Chemical structures of metabolites of kynurenine pathway.

consumption and T$_{reg}$ cells are activated by KP metabolites. Inhibition of IDO1 by small molecules hampers cancer cells to evade the immune system, favoring inflammatory response in the tumor microenvironment [18]. *In vitro* and *in vivo* pharmacological studies have supported this concept, showing that combination therapy between IDO1 inhibitors and chemotherapeutic drugs synergistically improves the efficacy of blocking tumor growth [19]. Although several chemical classes of IDO1 inhibitors have been reported in the literature and patent applications, only few compounds have progressed in clinical trials [20,21]. The stagnant rate of inhibitors progressing to clinical setting has been in part ascribed to challenges associated with a correct appraisal of IDO1 inhibition [22]. The relevance of a preferential binding of inhibitors to the

oxidized inactive form of IDO1 and/or to the reduced active form of the enzyme is also elusive in terms of pharmacological efficacy, cancer cell selectivity and off-target effects against other heme-containing proteins. Substrate inhibition at high concentrations of *L*-Trp (**1**) has been reported as potentially interfering with enzymatic assays aimed at investigating IDO1 inhibition kinetics of small molecules [23]. This observation was formerly explained by the presence of an accessory-binding site into IDO1 structure that may also host small-molecule enhancers of the catalytic activity [24–27]. However, more recent studies have demonstrated that substrate inhibition by *L*-Trp (**1**) is likely due to competition with oxygen in binding to heme cofactor [28,29]. Germane to this aspect, many IDO1 inhibitors reported in the literature have been described as endowed with noncompetitive or uncompetitive inhibition kinetics with respect to *L*-Trp (**1**) [3,30]. Crystallographic studies have shown that at least some of these compounds bind to the catalytic cleft of the enzyme [31–33]. Accordingly, their 'apparent' noncompetitive or uncompetitive profile is due to competition with binding of oxygen to heme, and not to the interaction with an accessory-binding site of the protein. In this framework, it is still questioned the mechanism of action of *D*-1-methyl-tryptophan (Indoximod, **6**, Figure 2) [34–37], which is currently in clinical trials as *bona fide* IDO1 inhibitor [38]. Likewise, concerns exist on whether inhibitors bearing quinone or iminoquinone moieties block the catalytic activity of IDO1 by a specific mechanism of action or rather by an unspecific redox-cycling mechanism [39,40].

Thermophoresis is the movement of biomolecular molecules in a temperature gradient and depends on size, charge and hydration shell. Typically upon ligand/protein interaction, at least one of these parameters
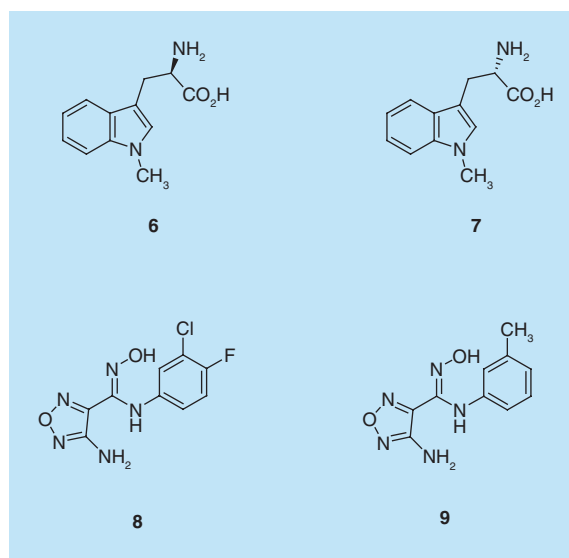
Figure 2. Chemical structures of competitive IDO1 inhibitors.

will change [41]. Dissociation constants (K_d) can thus be obtained analyzing the change of movement of the unbound molecule compared with the ligand-bound complex in a temperature gradient. Accordingly, herein we attempt to investigate binding properties of a selection of IDO1 inhibitors belonging to different categories of mechanism of action, by using MicroScale Thermophoresis (MST) analysis. Docking calculations were also performed to infer about the putative-binding modes of these inhibitors into the crystal structure of IDO1.

Methodology
MST analysis
Ligands were purchased from different vendors (Selleckchem, Munich, Germany; Sigma Aldrich, Milan, Italy; Molport, Riga, Latvia). Tryptanthrin derivative (**12**) was synthesized in our laboratory following the synthetic scheme already published in the literature [42]. Recombinant human IDO1 (rhIDO1) was purchased from Proteros. Experiments to assess K_d were conducted using Monolith NT.115 (NanoTemper Technologies, Munich, Germany). Fluorescence labeling of rhIDO1 was performed following the protocol for N-hydroxysuccinimide coupling of the dye NT647 (NanoTemper Technologies) to lysine residues. Briefly, 100 µl of a 20 µM solution of rhIDO1 protein in labeling buffer (130 mM $NaHCO_3$, 50 mM NaCl, pH 8.2) was mixed with 100 µl of 60 µM NT647-N-hydroxy-succinimide fluorophore (NanoTemper Technologies) and incubated for 30 min at room temperature in the dark. Unbounded fluorophores were removed by size-exclusion chromatography with MST buffer (50 mM TRIS, 150 mM NaCl, 10 mM $MgCl_2$, pH 7.4, 0.05% Tween20) as running buffer. The real concentration of each element of the sample, such as protein, heme group and RED dye and the degree of labeling were determined using extinction coefficient $\varepsilon 280 = 51,380$ $M^{-1\,cm-1}$ for rhIDO1, $\varepsilon 405 = 159,000$ $M^{-1\,cm-1}$ for rhIDO1 heme group and $\varepsilon 650 = 250,000$ $M^{-1\,cm-1}$ for NT647 fluorophore, with a correction factor (F_{corr}) of 0.028 at 280 nm, using $C_{prot} = [A280 – (A280 × Fcorr)/\varepsilon 280 × l]$ and the degree of labeling resulted between 0.6 and 0.8 throughout all labeling reactions.

The stability of NT647-rhIDO1 and unmodified rhIDO1 protein was checked using circular dichroism (Supplementary Table S1). Compound predilutions were prepared for MST experiments by 16-fold 1:1 serial dilutions in assay buffer containing 4% DMSO in PCR tubes supplied by NanoTemper Technologies to yield final volumes of 10 µl. NT647-rhIDO1 solution (10 µl) was added to each compound dilution and mixed to reach a final NT647-rhIDO1 concentration of 50 nM, including 2% DMSO, 2-mM DTT and a

reaction volume of 20 µl. These samples were loaded into 16 premium-coated capillaries and inserted in the chip tray of the MST instrument (Monolith NT.115) for thermophoresis analysis and the appraisal of K_d values. MST signals were recorded at MST 40% (compounds **6**, **7**, **9**, **11**, **12**, **13**). Compounds not providing a binding curve with a good S/N ratio at 40% (**8**, **10**) were tested at MST 80%. In both cases, 20% LED power was used. K_d values were calculated from compound concentration-dependent changes in normalized fluorescence (F_{norm}) of NT647-rhIDO1 after 21 s of thermophoresis for compounds tested at MST 40% and after 4 s for compounds tested at MST 80%. Each compound was tested in triplicate and the values reported were generated through the usage of *MO Affinity Analysis* software (NanoTemper Technologies); ($\pm$) K_d *confidence* (SD) are indicated next to K_d value. Specifically, confidence (SD) values define the range where the K_d falls with a 68% of certainty. Binding curves of protein fraction bound versus ligand concentration (nM) of tested compounds are shown in Supplementary Materials (Supplementary Figures 1–8).

Molecular docking
Ligands were prepared with *LigPrep 3.8* generating all ionization states at pH 7 ± 2. IDO1 crystal structure with the lowest resolution factor (Protein Data Bank [PDB] code: 5EK3, res. = 2.21 Å) was downloaded from the PDB. After deleting chain B, the Protein Preparation Wizard (Maestro 10.6, Schrödinger, Inc., NY, USA) tool was used to prepare chain A adding hydrogen atoms, setting ionization states at pH 7.0 and refining the structure by energy minimization. The oxidation state of the iron atom of the heme group was set to Fe^{3+} [31,43–44]. A grid box was generated with Glide 7.1 (Schrödinger, Inc., NY, USA), locating the center on the center of mass of the cocrystallized ligand (S, R)-**11** in 5EK3. The inner grid box was sized 12 × 12 × 12 Å. Docking studies were carried out using Glide 7.1 standard precision mode and storing the top five binding poses for each molecule. The best binding pose for each compound in terms of interaction energy (G-score, kcal/mol) [45–47] was selected in order to obtain a putative-binding mode.

Results & discussion
Competitive inhibitors
Analogs of *L*-Trp were among the first competitive inhibitors reported in the literature [48]. These compounds were designed with bioisosteric replacements of the indolic nitrogen or the N-methyl substitution at the indolic ring (1-Methyl-Trp). In particular, the racemic mixture of 1-Methyl-Trp was reported to inhibit IDO with a K_i of 34 µM. However, the *L*- (or

S, **7**) isomer proved more potent than the corresponding *D*- (or *R*, **6**) isomer, with the former showing 63% of inhibition at 100 μM and the latter having 12% of inhibition at 100 μM [49]. Of note, recent biochemical studies have evidenced that *L*-1-Methyl-Trp (**7**) is a slow substrate (hIDO1 k_{cat} = 0.027 ± 0.001 s⁻¹; K_M = 150 ± 11 μM) rather than a competitive inhibitor of the enzyme [50]. This observation suggests that *L*-1-Methyl-Trp (**7**) may bind to the oxygen-bound activated state of ferrous IDO1, leading to the production of N-formyl-methylkynurenine.

Despite its lower IDO1 inhibition potency in biochemical assay [49], *D*-1-Methyl-Trp (**6**) was found by some authors as endowed with superior *in vivo* anticancer activity and *in vitro* T-cell activation efficacy [34]. Although these observations have contributed to advance *D*-1-Methyl-Trp (**6**) in Phase I clinical trials as combination therapy with chemotherapeutic drugs for cancer, *L*-1-Methyl-Trp (**7**) is still employed as chemical probe to unravel biological functions of IDO1 as well as its therapeutic relevance in immunological and cancer disorders [35–37].

To get insights into binding properties of *L*-1-Methyl-Trp (**7**) and *D*-1-Methyl-Trp (**6**) to hIDO1, we determined K_d using MST. At this regard, it should be stressed that these K_d, and those reported hereinafter, refer to interactions with the inactive ferric (Fe^{3+}) form of hIDO1, which is the most stable in the adopted experimental conditions, and do not rule out that different K_d values for the same ligand may be associated to interactions with other forms of hIDO1, including the reduced ferrous (Fe^{2+}) state and/or oxygen-bound complex of the enzyme. As a result, a low K_d was found for *L*-1-Methyl-Trp (**7**, K_d = 1.38 ± 0.28 μM, Table 1, Supplementary Figure S1), whereas a higher K_d was determined for *D*-1-Methyl-Trp (**6**, K_d = 21.6 ± 2.8 μM, Supplementary Figure S2). The K_d value of *D*-1-Methyl-Trp (**6**), in particular, suggests for the first time that it may directly bind to the ferric inactive state of the enzyme. Of note, this observation

would be in agreement with the evidence that *D*-1-Methyl-Trp (**6**) is able to inhibit IDO1 catalytic activity in human and mouse dendritic cells [34]. Conversely, the poor inhibitory activity of *D*-1-Methyl-Trp (**6**) in biochemical assay would find tentative explanations in the inability of such ligand to prevent the reductive activation of IDO1 promoted by the ascorbic acid-methylene blue redox system, and/or the lack of binding affinity of *D*-1-Methyl-Trp (**6**) toward the reduced ferrous and/or oxygen-bound form of IDO1, with either events being at odds with *L*-1-Methyl-Trp (**7**).

Docking studies of these compounds into the inactive ferric form of IDO1 reveal top scored solutions with conserved binding poses. In the case of *L*-1-Methyl-Trp (**7**), the alpha amino group and carboxylic moiety make conserved hydrogen bonds with the propionate group of the heme cofactor and the carbonyl moiety of Gly262 (Figure 3A). The N-methyl indole ring is placed almost orthogonal to the heme plane, in a hydrophobic 'sandwich' composed by Tyr126 and Phe163. A similar binding mode is also observed for *D*-1-Methyl-Trp (Figure 3B). Remarkably, the similar docking score values of *L*-1-Methyl-Trp (**7**) and *D*-1-Methyl-Trp (**6**) into the catalytic cleft of hIDO1 are not able to explain the different K_d (Table 1). Although a flaw of the scoring function may well explain such observation, it is also possible that K_d of these ligands may be affected not only by interactions with residues of the catalytic pocket but also by interactions with other regions of hIDO1 such as the large unsolved loop located at the entrance of the catalytic site.

Hydroxyamidine-based compounds were also reported in the literature as potent competitive inhibitors of hIDO1, with preferential interactions to the active ferrous (Fe^{2+}) form of the enzyme [51]. Specifically, compounds **8** and **9** yielded IC_{50} of 0. 067 μM and 0.55 μM at inhibiting hIDO1 in biochemical assay, respectively. These inhibitors proved slightly more active in HeLa cell-based assays, with cellular IC_{50} of 0.019 μM for

Table 1. Dissociation constants and calculated binding energies of selected IDO1 inhibitors.			
Compound	Category	K_d ± SD (μM)	Gscore (kcal/mol)
6	Competitive	1.38 ± 0.28	-6.70
7	Competitive	21.6 ± 2.8	-6.71
8	Competitive	30.9 ± 2.7	-5.92
9	Competitive	33.4 ± 5.7	-5.83
10	Noncompetitive	45.3 ± 11.7	-6.71
11	Noncompetitive	3.30 ± 0.41	-7.41
12	Uncompetitive	0.97 ± 0.07	-6.01
13	Uncompetitive	790.6 ± 117.6	-4.58
K_d: Dissociation constant; SD: Confidence values (see 'Methods' for further details).			
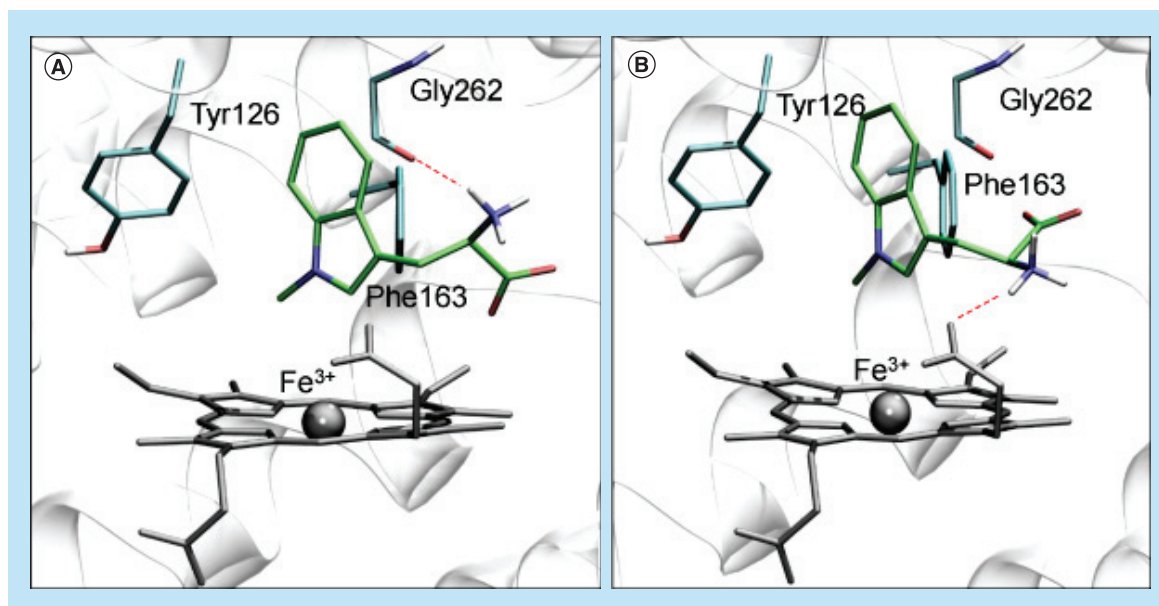
Figure 3. Docking studies of competitive inhibitors. Top scored binding poses of *L*-1-Methyl-Trp (A) and *D*-1-Methyl-Trp (B).

compound **8** and IC_{50} of 0.12 μM for compound **9.** MST experiments show high K_d for these inhibitors to hIDO1 (**8**, K_d = 30.9 ± 2.7 μM, Supplementary Figure S3; **9**, K_d = 33.4 ± 5.7 μM, Supplementary Figure S4). K_d and IC_{50} values are not directly comparable, with the former parameter encoding the binding event and the latter describing the functional activity. These diverging results are likely explained with the limit of the biophysical study in which the reduced catalytically active ferrous (Fe^{2+}) form of the enzyme cannot be stabilized with the addition of reducing agents that would potentially interfere with the MST assay. Hence, MST results indirectly support the preferential interaction of **8** and **9** to the ferrous (Fe^{2+}) form of the enzyme. Although the submicromolar potency of these molecules in biochemical assay is ascribed to their interaction with the active ferrous (Fe^{2+}) form of IDO1 [51], MST results pinpoint that **8** and **9** may also bind to the inactive ferric (Fe^{3+}) form of the enzyme at higher concentrations and in a range of potency that is remarkably similar to the K_d of *D*-1-Methyl-Trp. At this regard, it is worth noting that a high dose (75 mg/kg) of **8** was required to observe significant tumor growth inhibition in C57BL/6 mice bearing GM-CSF-secreting B16 melanoma. While the high dosage of **8** may be dictated by poor pharmacokinetic properties, the above observations raise questions about the potential contribution of the interaction to the inactive ferric form of hIDO1 to the *in vivo* antitumor efficacy of compound **8** and, more in general, of competitive inhibitors.

Results of docking studies of compounds **8** and **9** into the ferric form of hIDO1 are not in agreement with previous computational studies, which suggested that the

hydroxyamidine group coordinates the ferrous heme iron into the catalytic pocket of the enzyme. Although different adopted docking algorithms may explain this discrepancy, it is also possible that hydroxyamidine derivatives may use two alternative binding modes to interact with the active ferrous form of the enzyme or the inactive ferric form of IDO1. In the top scored binding pose of compound **8** (Figure 4A), the hydroxyamidine group makes a hydrogen bond with the propionate group of the heme cofactor. The phenyl moiety extends toward the entrance of the catalytic pocket, engaging residues Leu230, Phe226, Ile354 and Leu384 with hydrophobic contacts. The furazan moiety is placed into the hydrophobic pocket above the heme plane, being composed of residues Tyr126, Val130, Phe163 and Phe164. Its amino group interacts with the hydroxyl group of Ser167 making a hydrogen bond. In the top scored binding pose of compound **9** (Figure 4B), however, the phenyl and furazan moieties invert their relative position occupying the one the hydrophobic pocket of the other; inverting the pose, **9** loses the hydrogen bond with Ser167 but it binds to the carbonyl moiety of Gly262 and Leu234. In agreement with their equipotent K_d to ferric hIDO1, inhibitors **8** and **9** show similar docking score values in the top scored binding poses (Table 1).

Noncompetitive inhibitors

4-Phenylimidazole (4-PI, **10**, Figure 5) is one of the earlier noncompetitive inhibitors of IDO1 disclosed in the literature with a reported IC_{50} of 48 μM [52]. Compound **10** was used in crystallographic studies to solve the first ligand-bound crystal structure of the
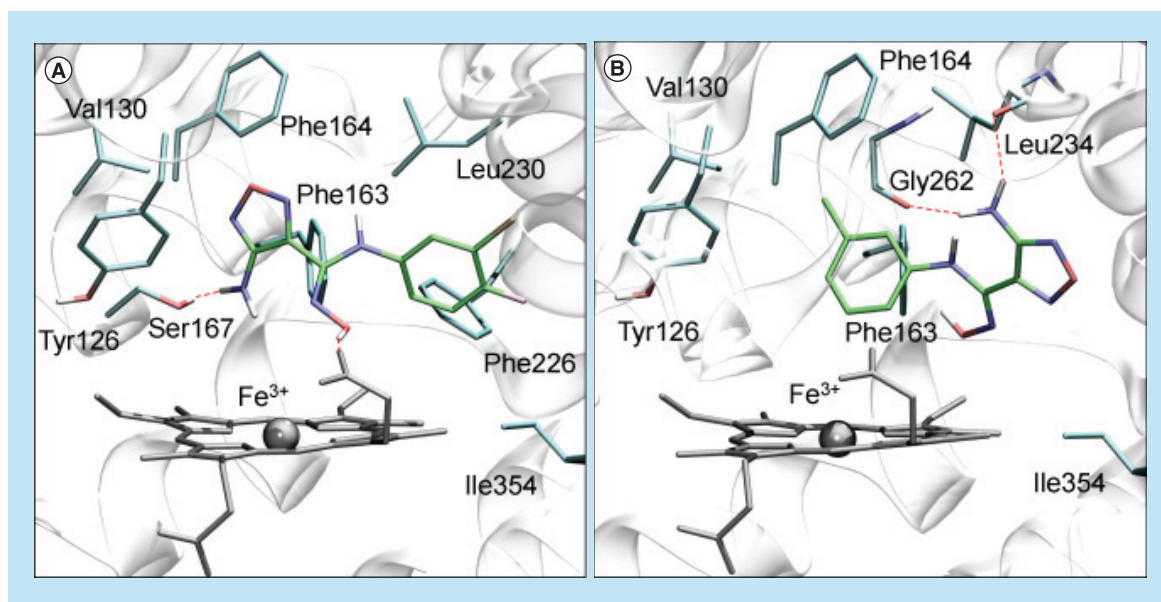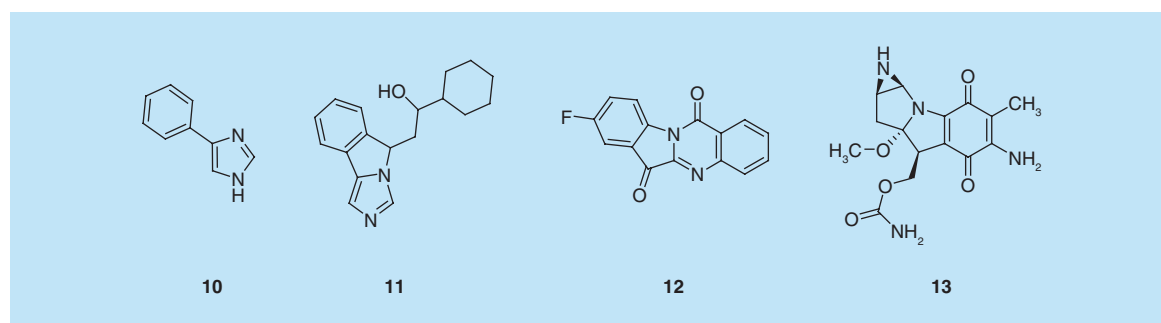
Figure 4. Docking studies of competitive inhibitors. Top scored binding poses of hydroxyamidine derivatives (**8, A**; **9, B**).

enzyme (PDB code: 2D0T), evidencing that the distal basic nitrogen atom of the imidazole ring is involved in a coordinative interaction with the sixth coordination site of the ferric heme of IDO1 [33]. This structure was instrumental to design 4-PI analogs and carry out structure–activity relationship studies that led to identify imidazoleisoindole derivatives as a chemical class of potent noncompetitive inhibitors of IDO1 [53]. In this study, we determined K_d to hIDO1 for 4-PI (**10**) and an imidazoleisoindole analog (**11**) that was recently solved in a crystallographic complex with hIDO1 (PDB code: 5EK3) [31]. MST analysis of compounds **10** and **11** reveals K_d of 45.3 ± 11.7 μM and K_d of 3.30 ± 0.41 μM to IDO1 (Table 1, Supplementary Figures S5, S6), respectively. At a first glance, these results are in agreement with the rank order of IC_{50} potency that is reported in the literature for 4-PI (**10**, IC_{50} = 48 μM) and **11** (IC_{50} = 0.1 μM or 0.038 μM) [31,54]. However, it cannot be ruled out that the imidazoleisoindole analog (**11**)

also binds to the reduced active ferrous (Fe^{2+}) state of hIDO1, with this latter interaction accounting for the nanomolar inhibition potency in biochemical assay. A scenario reported in the literature for another noncompetitive inhibitor of IDO1, namely Norharman, which binds to both the ferric and ferrous form of the enzyme with comparable K_d (~10 μM) [52].

Docking studies of the imidazole derivatives **10** and (*S, R*)-**11** are in different agreement with the relative experimental crystal structures (Supplementary Tables S2, S3). Specifically, docking of 4-PI (**10**) is unable to provide top scored solutions consistent with its experimental crystallographic complex of IDO1 (Root-mean-square deviation [RMSD] = 2.68 Å, Figure 6A). Reason of such a failure has been discussed by us elsewhere and is out of the scope of the present work [55]. Conversely, results of docking of the imidazoleisoindole analog (*S, R*)-**11** into the catalytic cleft of IDO1 are in good agreement with

Figure 5. Chemical structures of noncompetitive IDO1 inhibitors (10, 11), and uncompetitive IDO1 inhibitors (12, 13).
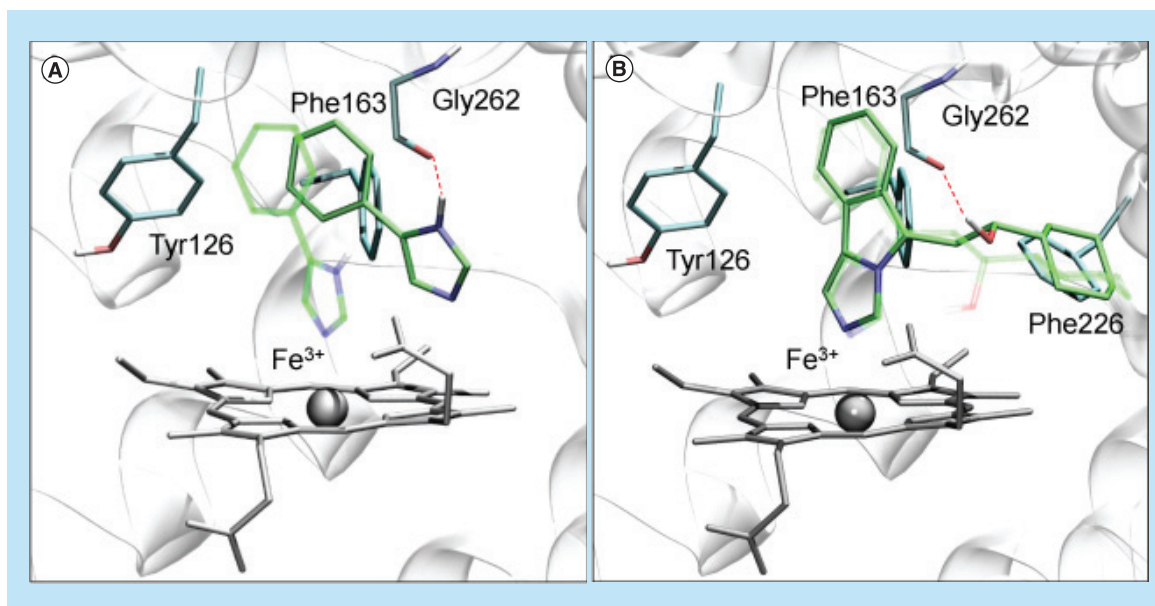
Figure 6. Docking studies of noncompetitive inhibitors. Top scored binding poses of noncompetitive IDO1 inhibitors (**10, A; 11, B**). Experimental binding poses are shown in transparency.

the experimental crystallographic complex (RMSD = 0.98 Å, Figure 6B). In particular, (*S*, *R*)-**11** places the basic nitrogen atom of the imidazole moiety within a distance (<3 Å) and angle (about 180° considering the iron and the nitrogen of the underlying His346) that are suitable for iron coordination. The hydroxyl group takes contact through H-bond with Gly262 (or the heme propionate group in other less energetic favored solutions), the three fused rings are packed with Tyr126 and Phe163 and the cyclohexyl moiety engages Phe226 with hydrophobic contacts.
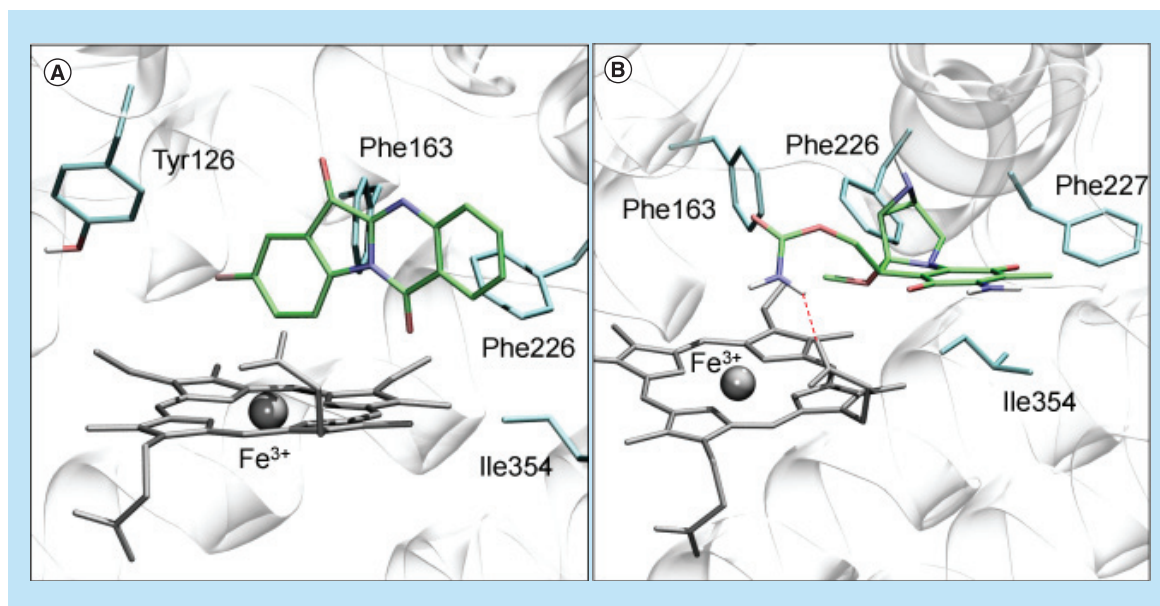
Uncompetitive inhibitors

Uncompetitive inhibitors are defined as ligands that bind to the enzyme/substrate complex with the maximal affinity. Although the substrate (*L*-Trp) was not added to the composition of the MST assay solution due to potential interference with the biophysical assay, we still pursued the exploration of the potential target engagement by MST of these compounds, providing an assessment of their binding properties to the substrate unbound form of IDO1.

Tryptanthrin derivatives were reported as potent uncompetitive inhibitors of IDO1 [42]. Among these, compound **12** proved low micromolar K_i (= 0.161 µM) and IC_{50} (= 0.534 µM) against rhIDO1, while a more potent inhibitory activity was found in HEK 293 cells (IC_{50} = 0.023 µM) expressing human IDO1. Compound **12** was able to inhibit tumor growth in Lewis lung cancer cell-bearing mice, as well as reduced Foxp3+ T_{reg} cells in the same animal model. At odds with its *in vitro* and *in vivo* potency, however, compound **12** was

found with a poor dissociation constant to IDO1 in a surface plasma resonance study (K_d = 46.8 µM) reported by the same authors. However, the MST analysis pinpoints a higher affinity of **12**, providing a K_d of 0.97 ± 0.07 µM (Table 1, Supplementary Figure S7), namely a value that is more in agreement with the reported biochemical and cellular activity of the compound. These different results of surface plasma resonance and MST are likely due to the fact that the former assay implies the immobilization of the enzyme, whereas the latter is carried out with the free enzyme in solution. As a consequence, the immobilization protocol may have affected the conformational properties of IDO1, leading to assess a poor dissociation constant of the ligand in surface plasma resonance assay.

Despite none of the trypthantrin derivatives has been cocrystallized with IDO1 and no binding mode has been proposed, we have defined its putative-binding mode through its best solution in 5EK3 (Figure 7A). In particular, the fluorine-substituted phenyl goes into the hydrophobic cleft composed by Tyr126 and Phe163, while the opposite no-substituted phenyl is placed toward the entry channel and takes contact with Phe226 and Ile354.

Mitomycin-C (**13**) is a natural product that has been used as chemotherapeutic agent for several types of cancer [56–58]. It was reported as uncompetitive inhibitor of IDO1 with a K_i of approximately 25 µM [24]. According to its mechanism of inhibition, the authors proposed that mitomycin-C (**13**) binds to the substrate-bound complex of IDO1, interacting with an accessory site that is shaped upon substrate

Figure 7. Docking studies of uncompetitive inhibitors. Top scored binding poses of uncompetitive IDO1 inhibitors (**12**, **A**; **13**, **B**).

binding to the enzyme. Herein, we assessed the dissociation constant of mitomycin-C (**13**) to IDO-1 by the MST analysis. Although it was not possible to obtain a saturated-binding curve for compound **13** (Supplementary Figure S8) due to limitation of compound solubility, a binding event was observed with an approximate K_d value of 790.6 ± 117.6 μM (Table 1). Again, it should be stressed that such a high micromolar K_d value is obtained against the substrate unbound ferric form of the enzyme to have an assessment of target engagement. Accordingly, MST does highlight the presence of a low-affinity accessory site for mitomycin-C (**13**) in the free enzyme, and it does not rule out that *L*-Trp binding into the catalytic site of IDO1 may adjust the shape of the accessory site to allow for hosting the large structural features of **13**, thereby enabling a stronger inhibition constant with maximal affinity for the natural compound. This observation combines with the presence of a quinone moiety in the chemical structure of mitomycin-C (**13**). Other natural products bearing quinone groups have been reported as potent IDO1 inhibitors [59,60]. Grounding on this observation, screening of commercially available compounds containing a quinone group resulted in the identification of several potent IDO1 inhibitors [40]. However, the inhibitory activity of these compounds has been debated, questioning whether they adopt a specific mechanism of action to inhibit IDO1 or act through an unspecific redox-cycling mechanism. The inhibitory potency of mitomycin-C (**13**) in biochemical assay may thus arise from the coupling of specific binding event to IDO1 and unspecific redox-cycling mechanism.

Results of docking studies of mitomycin-C (**13**) are in accordance with the assessed K_d. Indeed, compound **13** shows the worst *g-score* among the studied compounds. Because of its larger size, mitomycin-C (**13**) does not fit the catalytic cleft above the heme group, but it is placed toward the entry channel (Pocket B, Figure 7B). The bad score is in line with the uncompetitive mechanism of inhibition of mitomycin-C (**13**), which indeed suggests maximal interaction of **13** with the enzyme/substrate complex. Hence, a different conformation of IDO1 is required for docking studies. Specifically, the structure of the enzyme with the substrate and/or oxygen bound to the catalytic cleft for which crystallographic data are not still available.

Conclusion & future perspective

In this study, we have used the MST analysis to quantitatively assess the binding properties of distinct categories of inhibitors to IDO1, including competitive, noncompetitive and uncompetitive inhibitors. Germane to competitive inhibitors, results pinpoint specific binding properties of *L*-1-Methyl-Trp (**8**) and *D*-1-Methyl-Trp (**7**) to the ferric form of IDO1, with the former showing a stronger dissociation than the latter stereoisomer. Although the pharmacological relevance of a specific interaction of inhibitors toward the inactive ferric form of IDO1 and/or the active ferrous form of the enzyme is still elusive, these data support an IDO1-mediated anticancer activity for *D*-1-Methyl-Trp (**7**), a *bona fide* IDO1 inhibitor that is currently in clinical settings as single-agent therapy (Clinical Trials Id.: NCT00739609,

NCT00567931, NCT01560923) or in combination therapies (Clinical Trials Id.: NCT02835729, NCT02502708, NCT02077881) for cancer disease. K_d of noncompetitive inhibitors, such as 4-PI (**10**) and imidazoleisoindole analog (**11**), are in overall agreement with their biochemical and cellular IC_{50} values. However, the low-micromolar K_d value of **11** is somehow higher than what expected from its nanomolar inhibition potency reported in the literature. A poor dissociation constant in the high-micromolar range is also found for mitomycin-C (**13**), a known natural anticancer compound that has been reported as uncompetitive inhibitor with the ability to bind to the substrate-bound complex of IDO1. Taken together the data presented in this work provide further insights into the binding properties of inhibitors to the ferric form of IDO1, offering novel clues for future development of more efficient IDO1 inhibitors. Moreover, our results support the use of MST analysis as biophysical tool that, combining with available biochemical and cellular assays of IDO1, may overcome issues related to the undesired identification of lead compounds with unspecific mechanism of inhibition.

Supporting information available

Supplementary Table S1 contains results from Circular Dichroism study to check the stability of IDO1 and NT647-IDO1. Supplementary Tables S2 and S3 report top scored solutions from docking studies of 4-PI (**10**) and (*S*, *R*)-**11** into the catalytic

---

Summary points

Background
- Indoleamine 2,3-dioxygenase 1 (IDO1) is a heme-containing enzyme that catalyzes the oxidative cleavage of *L*-Trp (**1**) in the rate-limiting step of the kynurenine pathway.
- IDO1 is overexpressed in many human cancer cells and in dendritic cells presenting tumor antigens.
- IDO1 acts propelling cancer cell growth by creating an immune-privileged tolerogenic microenvironment in which cytotoxic T cells are starved by *L*-Trp consumption and T_{reg} cells are activated by the metabolites of kynurenine pathways.
- Inhibitors of IDO1 hamper cancer cells to evade the immune system, favoring inflammatory response in the tumor microenvironment.
- Although an ever-increasing number of IDO1 inhibitors are disclosed in the literature and patent applications, there is a stagnant rate of compounds advancing into clinical setting due to challenges associated with a correct appraisal of IDO1 inhibition.
- The mechanism of action of the anticancer drug candidate *D*-1-Methyl-Trp (Indoximod, **6**) is still questioned as to whether it is truly mediated by IDO1.

Aim
- Binding properties of a selection of different inhibitors to IDO1 are herein investigated using the MicroScale Thermophoresis (MST) analysis.
- Docking calculations are performed to infer about the putative-binding modes of selected IDO1 inhibitors.

Method
- Thermophoresis is the movement of biomolecular molecules in a temperature gradient that depends on size, charge and hydration shell typically changing upon ligand/protein interaction.

Results & discussion
- Both stereoisomers *L*-1-Methyl-Trp (**7**, K_d = 1.38 ± 0.28 μM) and *D*-1-Methyl-Trp (**6**, K_d = 21.6 ± 2.8 μM) bind to recombinant human IDO1, suggesting a direct interaction of Indoximod with the ferric state of the enzyme.
- In agreement with their preferential interaction with the reduced active form of IDO1, competitive hydroxyamidine inhibitors (**8**, **9**) bind to the inactive state of the enzyme with high-micromolar dissociation constants (**8**, K_d = 30.9 ± 2.7 μM; **9**, K_d = 33.4 ± 5.7 μM).
- MST experiments of noncompetitive inhibitors 4-PI (**10**) and imidazoleisoindole analog (**11**) yield K_d values of 45.3 ± 11.7 and 3.30 ± 0.41 μM, respectively.
- The uncompetitive inhibitor **12** provides the most potent K_d value (0.97 ± 0.07 μM) determined in this study.
- Mitomycin-C (**13**) binds to IDO-1 with an approximate K_d value of 790.6 ± 117.6 μM, suggesting that other factors may account for the reported inhibition potency of this natural product.

Conclusion & future perspective
- The MST analysis pinpoints specific binding properties of *L*-1-Methyl-Trp (**8**) and *D*-1-Methyl-Trp (**7**) to the ferric form of IDO1.
- Results support an IDO1-mediated anticancer activity of *D*-1-Methyl-Trp (Indoximod, 7).
- Knowledge on K_d of selected inhibitors to IDO1 offer novel clues for the design and development of more efficient inhibitors.
- MST is a valuable biophysical tool that may overcome issues related to the undesired identification of compounds with unspecific mechanism of IDO1 inhibition.

cleft of IDO1. Supplementary Figures S1–S8 show MST-binding curves of protein fraction bound versus ligand concentration (nM) of tested compounds. To view the supplementary data that accompany this paper please visit the journal website at: www.future-science/doi/full/10.4155/FMC-2017-0022.

References

Papers of special note have been highlighted as: • of interest;
•• of considerable interest

1   Murray PJ. Amino acid auxotrophy as a system of immunological control nodes. *Nat. Immunol.* 17(2), 132–139 (2015).

2   Mcgaha TL, Metz R, Prendergast GC, Mellor AL. Amino acid catabolism: a pivotal regulator of innate and adaptive immunity. *Immunol. Rev.* 249, 135–157 (2012).

3   Macchiarulo A, Camaioni E, Nuti R, Pellicciari R. 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. *Amino Acids.* 37, 219–229 (2009).

4   Dounay AB, Tuttle JB, Verhoest PR. Challenges and opportunities in the discovery of new therapeutics targeting the kynurenine pathway. *J. Med. Chem.* 58, 9421–9437 (2015).

5   Yeung AWS, Terentis AC, King NJC, Thomas SR. Role of indoleamine 2,3-dioxygenase in health and disease. *Clin. Sci. (Lond).* 129(7), 601–672 (2015).

6   Greco F, Coletti A, Camaioni E *et al.* The Janus-faced nature of IDO1 in infectious diseases: challenges and therapeutic opportunities. *Future Med. Chem.* 8(1), 39–54 (2016).

7   Fallarino F, Grohmann U, Puccetti P. Indoleamine 2,3-dioxygenase: from catalyst to signaling function. *Eur. J. Immunol.* 42(8), 1932–1937 (2012).

8   Orabona C, Pallotta M. Different partners, opposite outcomes: a new perspective of the immunobiology of indoleamine 2,3-dioxygenase. *Mol. Med.* 18(5), 1 (2012).

9   Pallotta MT, Orabona C, Volpi C *et al.* Indoleamine 2,3-dioxygenase is a signaling protein in long-term tolerance by dendritic cells. *Nat. Immunol.* 12(9), 870–880 (2011).

•• **A novel signaling function is discovered for indoleamine 2,3-dioxygenase 1 (IDO1) that is independent from the catalytic activity.**

10 Orabona C, Pallotta MT, Volpi C *et al.* SOCS3 drives proteasomal degradation of indoleamine 2,3-dioxygenase (IDO) and antagonizes IDO-dependent tolerogenesis. *Proc. Natl Acad. Sci. USA* 105(52), 20828–20833 (2008).

11 Pfefferkorn ER. Interferon gamma blocks the growth of Toxoplasma gondii in human fibroblasts by inducing the host cells to degrade tryptophan. *Proc. Natl Acad. Sci. USA* 81(3), 908–912 (1984).

12 Moffett JR, Namboodiri MA. Tryptophan and the immune response. *Immunol. Cell Biol.* 81(4), 247–265 (2003).

13 A-B Badawy A, Namboodiri AM, Moffett JR. The end of the road for the tryptophan depletion concept in pregnancy and infection. *Clin. Sci.* 130, 1327–1333 (2016).

14 Uyttenhove C, Pilotte L, Théate I *et al.* Evidence for a tumoral immune resistance mechanism based on tryptophan degradation by indoleamine. *Nat. Med.* 9(10), 1269–1274 (2003).

•• **Elevated levels of IDO1 in human cancers are linked to the tumor immuno-editing functions of the enzyme.**

15 Lob S, Konigsrainer A, Rammensee HG, Opelz G, Terness P. Inhibitors of indoleamine-2,3-dioxygenase for cancer therapy: can we see the wood for the trees? *Nat. Rev. Cancer* 9(6), 445–452 (2009).

16 Sheridan C. IDO inhibitors move center stage in immuno-oncology. *Nat. Publ. Gr.* 33(4), 321–322 (2015).

17 Godin-Ethier J, Hanafi LA, Piccirillo CA, Lapointe R. Indoleamine 2,3-dioxygenase expression in human cancers: clinical and immunologic perspectives. *Clin. Cancer Res.* 17(22), 6985–6991 (2011).

18 Moon YW, Hajjar J, Hwu P, Naing A. Targeting the indoleamine 2,3-dioxygenase pathway in cancer. *J. Immunother. Cancer* 3, 51–61 (2015).

19 Muller AJ, DuHadaway JB, Donover PS, Sutanto-Ward E, Prendergast GC. Inhibition of indoleamine 2,3-dioxygenase, an immunoregulatory target of the cancer suppression gene Bin1, potentiates cancer chemotherapy. *Nat. Med.* 11(3), 312–319 (2005).

20 Soliman HH, Jackson E, Neuger T *et al.* A first in man Phase I trial of the oral immunomodulator, indoximod, combined with docetaxel in patients with metastatic solid tumors. *Oncotarget* 5(18), 8136–8146 (2014).

21 Vacchelli E, Aranda F, Eggermont A *et al.* Trial watch: IDO inhibitors in cancer therapy. *Oncoimmunology* 3(10), e957994 (2014).

22 Röhrig UF, Majjigapu SR, Vogel P, Zoete V, Michielin O. Challenges in the discovery of indoleamine 2,3-dioxygenase 1 (IDO1) inhibitors. *J. Med. Chem.* 58(24), 9421–9437 (2015).

•• **Challenges associated to the discovery and development of IDO1 inhibitors are elegantly reviewed.**

23 Sono M, Taniguchi T, Hayaishig O. Indoleamine 2,3-dioxygenase: equilibrium studies of the tryptophan binding to the ferric, ferrous and co-bound enzymes. *J. Biol. Chem.* 255(4), 1339–1345 (1980).

•   **First evidence of substrate inhibition at high concentration of *L*-Tryptophan is reported.**

24   Lu C, Lin Y, Yeh S-R. Inhibitory substrate binding site of human indoleamine 2,3-dioxygenase. *J. Am. Chem. Soc.* 131(36), 12866–12867 (2009).

25   Nickel E, Nienhaus K, Lu C, Yeh S, Nienhaus GU. Ligand and substrate migration in human indoleamine. *J. Biol. Chem.* 284(46), 31548–31554 (2009).

26   Lu C, Lin Y, Yeh S. Spectroscopic studies of ligand and substrate binding to human. *Biochemistry* 49, 5028–5034 (2010).

27   Macchiarulo A, Nuti R, Bellocchi D, Camaioni E, Pellicciari R. Molecular docking and spatial coarse graining simulations as tools to investigate substrate recognition, enhancer binding and conformational transitions in indoleamine-2,3-dioxygenase (IDO). *Biochim. Biophys. Acta* 1774, 1058–1068 (2007).

28   Efimov I, Basran J, Sun X *et al.* The mechanism of substrate inhibition in human indoleamine 2,3-dioxygenase. *J. Am. Chem. Soc.* 134(6), 3034–3041 (2012).

29   Weber B, Nickel E, Horn M, Nienhaus K, Nienhaus GU. Substrate inhibition in human indoleamine 2,3-dioxygenase. *J. Phys. Chem. Lett.* 5(4), 756–761 (2014).

30   Jiang T, Sun Y, Yin Z, Feng S, Sun L, Li Z. Research progress of indoleamine 2,3-dioxygenase inhibitors. *Future Med. Chem.* 7(2), 185–201 (2015).

31   Peng Y-H, Ueng S-H, Tseng C-T *et al.* Important hydrogen bond networks in indoleamine 2,3-dioxygenase 1 (IDO1) inhibitor design revealed by crystal structures of imidazoleisoindole derivatives with IDO1. *J. Med. Chem.* 59, 282–293 (2016).

32   Tojo S, Kohno T, Tanaka T *et al.* Crystal structures and structure – activity relationships of imidazothiazole derivatives as IDO1 inhibitors. *Med. Chem. Lett.* 5, 1119–1123 (2014).

33   Sugimoto H, Oda S, Otsuki T, Hino T, Yoshida T, Shiro Y. Crystal structure of human indoleamine 2,3-dioxygenase: catalytic mechanism of O2 incorporation by a heme-containing dioxygenase. *Proc. Natl Acad. Sci. USA* 103(8), 2611–2616 (2006).

•   **First crystal structure of IDO1 bound to a noncompetitive inhibitor (4-PI).**

34   Hou DY, Muller AJ, Sharma MD *et al.* Inhibition of indoleamine 2,3-dioxygenase in dendritic cells by stereoisomers of 1-methyl-tryptophan correlates with antitumor responses. *Cancer Res.* 67(2), 792–801 (2007).

35   Lob S, Konigsrainer A, Schafer R, Rammensee H, Opelz G, Terness P. Levo- but not dextro-1-methyl tryptophan abrogates the IDO activity of human dendritic cells Levo- but not dextro-1-methyl tryptophan abrogates the IDO activity of human dendritic cells. *Blood* 111(4), 2152–2155 (2007).

36   Qian F, Villella J, Wallace PK *et al.* Efficacy of levo-1-methyl tryptophan and dextro-1-methyl tryptophan in reversing indoleamine-2,3-dioxygenase-mediated arrest of T-cell proliferation in human epithelial ovarian cancer. *Cancer Res.* 69(13), 5498–5504 (2009).

37   Lim J, Lee S, Park G, Choi EY, Min C. Inhibition of indoleamine 2,3-dioxygenase by stereoisomers of 1-methyl tryptophan in an experimental graft-versus-tumor model. *Exp. Hematol.* 42(10), 862–866 (2014).

38   Soliman HH, Minton SE, Han HS *et al.* A Phase I study of indoximod in patients with advanced malignancies. *Oncotarget* 7(16), 22928–22938 (2016).

39   Röhrig UF, Majjigapu SR, Chambon M *et al.* Detailed analysis and follow-up studies of a high-throughput screening for indoleamine 2,3-dioxygenase 1 (IDO1) inhibitors. *Eur. J. Med. Chem.* 84, 284–301 (2014).

40   Kumar S, Malachowski WP, Duhadaway JB *et al.* Indoleamine 2,3-dioxygenase is the anticancer target for a novel series of potent naphthoquinone-based inhibitors. *J. Med. Chem.* 51, 1706–1718 (2008).

41   Duhr S, Braun D. Why molecules move along a temperature gradient. *Proc. Natl Acad. Sci. USA* 103(52), 19678–19682 (2006).

•   **The principles of MicroScale Thermophoresis are discussed.**

42   Yang S, Li X, Hu F *et al.* Discovery of tryptanthrin derivatives as potent inhibitors of indoleamine 2, 3-dioxygenase with therapeutic activity in LLC tumor-bearing mice. *J. Med. Chem.* 56, 8321–8331 (2013).

43   Schrödinger Release 2016–2: LigPrep, version 3.8. Schrödinger, LLC, New York, NY, USA (2016).

44   Schrödinger Release 2016–2: Maestro, version 10.6. Schrödinger, LLC, New York, NY, USA (2016).

45   Friesner RA, Halgren TA, Banks JL *et al.* Glide: a new approach for rapid, accurate docking and scoring. *J. Med. Chem.* 47(7), 1739–1759 (2004).

46   Friesner RA, Murphy RB, Repasky MP *et al.* Extra precision glide: docking and scoring incorporating a model of hydrophobic enclosure for protein-ligand complexes. *J. Med. Chem.* 49(21), 6177–6196 (2006).

47   Small-Molecule Drug Discovery Suite 2016–2: Glide, version 7.1. Schrödinger, LLC, New York, NY, USA (2016).

48   Cady SG, Sono M. 1-Methyl-DL-tryptophan, beta-(3-benzofuranyl)-DL-alanine (the oxygen analog of tryptophan), and beta-[3-benzo(b)thienyl]-DL-alanine (the sulfur analog of tryptophan) are competitive inhibitors for indoleamine 2,3-dioxygenase. *Arch. Biochem. Biophys.* 291, 326–333 (1991).

49   Peterson AC, Migawa MT, Martin MM. Evaluation of functionalized tryptophan derivatives and related compounds as competitive inhibitors of Indoleamine 2,3-dioxygenase. *Med. Chem. Res.* 3, 531–544 (1994).

50   Chauhan N, Thackray SJ, Rafice SA *et al.* Reassessment of the reaction mechanism in the heme dioxygenases. *J. Am. Chem. Soc.* 131(12), 4186–4187 (2009).

51   Yue EW, Douty B, Wayland B *et al.* Discovery of potent competitive inhibitors of indoleamine 2,3-dioxygenase with *in vivo* pharmacodynamic activity and efficacy in a mouse melanoma model. *J. Med. Chem.* 52(23), 7364–7367 (2009).

52   Sono M, Cady SG. Enzyme kinetic and spectroscopic studies of inhibitor and effector interactions with indoleamine 2,3-dioxygenase. 1. Norharman and 4-phenylimidazole binding to the enzyme as inhibitors and heme ligands. *Biochemistry* 28(13), 5392–5399 (1989).

53   Kumar S, Jaller D, Patel B *et al.* Structure based development of phenyl-imidazole-derived inhibitors of indoleamine 2,3-dioxygenase. *J. Med. Chem.* 51(16), 4968–4977 (2011).

54 Seegers N, van Doornmalen AM, Uitdehaag JCM, de Man J, Buijsman RC, Zaman GJR. High-throughput fluorescence-based screening assays for tryptophan-catabolizing enzymes. *J. Biomol. Screen.* 19(9), 1266–1274 (2014).

55 Greco FA, Bournique A, Coletti A *et al.* Docking studies and molecular dynamic simulations reveal different features of IDO1 structure. *Mol. Inform.* 35(8), 449–459 (2016).

56 Crooke ST, Bradner WT. Mitomycin C: a review. *Cancer Treat. Rev.* 3(3), 121–139 (1976).

57 Verweij J, Pinedo HM. Mitomycin C: mechanism of action, usefulness and limitations. *Anticancer Drugs* 1(1), 5–13 (1990).

58 Zargar H, Aning J, Ischia J, So A, Black P. Optimizing intravesical mitomycin C therapy in non-muscle-invasive bladder cancer. *Nat. Rev. Urol.* 11(4), 220–230 (2014).

59 Pereira A, Vottero E, Roberge M, Mauk AG, Andersen RJ. Indoleamine 2,3-dioxygenase inhibitors from the northeastern pacific marine hydroid Garveia annulata. *J. Nat. Prod.* 69(10), 1496–1499 (2006).

60 Brastianos HC, Vottero E, Patrick BO *et al.* Exiguamine A, an indoleamine-2,3-dioxygenase (IDO) inhibitor isolated from the marine sponge Neopetrosia exigua. *J. Am. Chem. Soc.* 128(50), 16046–16047 (2006).