

Challenges in the Discovery of Indoleamine 2,3-Dioxygenase 1 (IDO1) Inhibitors

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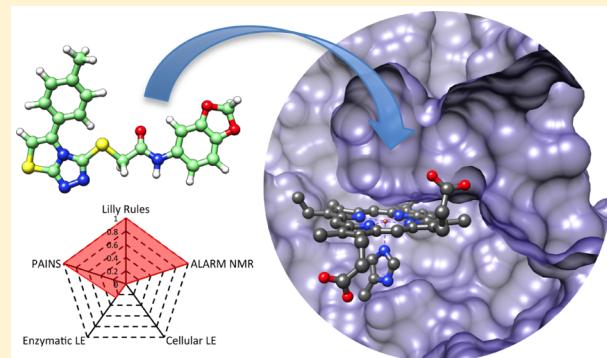
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ABSTRACT: Since the discovery of indoleamine 2,3-dioxygenase 1 (IDO1) as an attractive target for anticancer therapy in 2003, the search for inhibitors has been intensely pursued both in academia and in pharmaceutical companies. Many novel IDO1 inhibitor scaffolds have been described, and a few potent compounds have entered clinical trials. However, a significant number of the reported compounds contain problematic functional groups, suggesting that enzyme inhibition could be the result of undesirable side reactions instead of selective binding to IDO1. Here, we describe issues in the employed experimental protocols, review and classify reported IDO1 inhibitors, and suggest different approaches for confirming viable inhibitor scaffolds.



INTRODUCTION

Cancer immunotherapy is currently entering an exciting new era because of the efficacy of immune checkpoint inhibitors,¹ adoptive cell therapy with tumor infiltrating lymphocytes,² and chimeric antigen receptor T-cell therapy.³ It has been widely recognized that these therapies as well as more traditional approaches such as chemotherapy and radiotherapy could benefit from combination with a strategy to overcome tumor-induced immunosuppression.^{4–6} A central role in immune escape has been attributed to the kynurenine pathway of tryptophan (Trp) metabolism,^{7–9} which leads to the depletion of tryptophan and to the production of kynurenine metabolites, both responsible for local immunosuppression.^{10–12} Indoleamine 2,3-dioxygenase 1 (IDO1, EC 1.13.11.52), which catalyzes the initial and rate-limiting step of the kynurenine pathway, is expressed by tumor cells to escape a potentially effective immune response,^{10,13–15} and high IDO1 expression is associated with poor prognosis in a variety of cancer types.¹⁶ In vitro and in vivo studies demonstrate that administration of an IDO1 inhibitor improves the efficacy of therapeutic vaccination, chemotherapy, or radiationtherapy.^{13,17–20} Recently, the functionally related enzyme tryptophan 2,3-dioxygenase (TDO, EC 1.13.11.11) has been shown to play a complementary role in different types of cancer.^{21,22} In contrast, the role of the IDO1 parologue indoleamine 2,3-dioxygenase 2 (IDO2)^{23,24} remains less clear because of its very low Trp degradation activity.^{12,25–28}

IDO1 is a heme enzyme that catalyzes the oxidation of L-Trp to N-formyl kynurenine (NFK) by the incorporation of

molecular oxygen and the cleavage of the pyrrole ring of the substrate. The crystal structures of human IDO1^{29,30} show one binding pocket in the distal heme site (pocket A, Figure 1A), connected to a second pocket toward the entrance of the active site (pocket B).³¹ Intense efforts to develop IDO1 inhibitors are ongoing in academia and in pharmaceutical companies and yielded different active scaffolds. Two compounds have entered clinical trials, the hydroxyamidine 1 (INCB024360, epacadostat)^{18,32,33} developed by Incyte Corporation and more recently the imidazole NLG919 (structure not disclosed)^{34,35} from NewLink Genetics. 1-Methyl-D-tryptophan (D1MT, indoximod) from NewLink Genetics is also undergoing clinical trials as a kynurenine pathway inhibitor, but at variance with the L enantiomer³⁶ it is not an IDO1 inhibitor and its mechanism of action is debated.^{14,37–40} Other pharma companies with IDO1 or TDO inhibitors in their pipeline⁴¹ at present or in the past are Amgen,⁴² Bristol-Myers Squibb,^{43–46} Curadev,^{47,48} Dainippon Sumitomo Pharma Corporation,³⁰ Iomet Pharma,⁴⁹ iTeos Therapeutics,⁵⁰ and Vertex Pharmaceuticals.⁵¹

IDO1 inhibitory properties have been attributed to thousands of compounds according to the scientific and patent literature. However, a significant number of these compounds may show IDO1 inhibitory activity through unspecific mechanisms. While the problem of promiscuous molecules is a general one and receives increasing attention,⁵² it is very pronounced for IDO1

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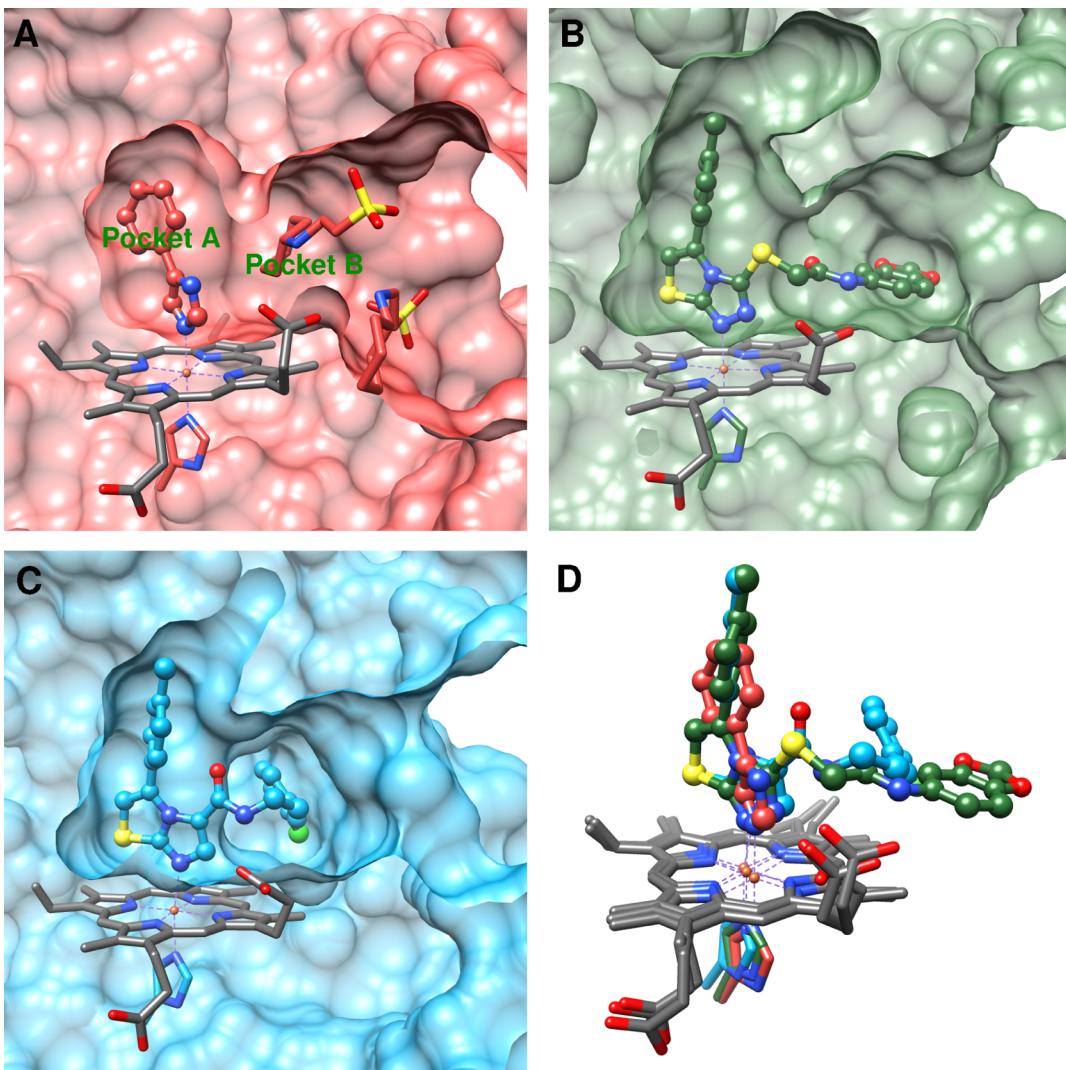


Figure 1. X-ray structures of IDO1.^{29,30} Only the active site is shown, displaying the protein in surface representation, heme and His346 in stick representation, and the ligand in ball and stick representation. Heteroatoms are colored as follows: blue (nitrogen), red (oxygen), yellow (sulfur), green (chloride), orange (iron). (A) Structure 2D0T bound to 4PI and two molecules of CHES buffer (displayed in stick representation).²⁹ (B) Structure 4PK5 bound to compound 2.³⁰ (C) Structure 4PK6 bound to imidazothiazole derivative 13b from ref 30. (D) Superposition of the X-ray structures 2D0T, 4PK5, and 4PK6, showing only the heme-bound groups.

because of its properties as a redox-sensitive heme protein and some features of commonly employed assays. Here, we first describe critical issues in experimental protocols before reviewing and classifying reported IDO1 inhibitors. Finally, we suggest different approaches for confirming viable inhibitor scaffolds.

■ BACKGROUND

Enzymatic Assay. Table 1 lists the common composition of the incubation medium used to determine IDO1 inhibitory activity of small organic molecules (modified from ref 53).

Redox Reactions. In the active form of IDO1, the heme iron is in its reduced ferrous state. Formally, the catalytic cycle of IDO1 does not alter the heme oxidation state, but as IDO1 is prone to autoxidation, a reductant is necessary to maintain enzyme activity. Cytochrome *b*₅ and cytochrome P450 reductase with NADPH have been suggested to be responsible for IDO1 reduction *in vivo*.^{54–56} However, in the usual enzymatic assay protocol used to determine IDO1 inhibition, purified ferric IDO1 is reduced by ascorbic acid, necessitating methylene blue

as electron carrier.⁵⁷ Catalase is added to avoid inhibition of IDO1 by H₂O₂.⁵⁸ A more physiological enzymatic assay protocol using cytochrome P450 reductase/NADPH and cytochrome *b*₅ as reductants has been described⁵⁶ but not reportedly been used by others, probably because of higher costs and reduced ease of manipulation.

Over the past decade many redox-cycling compounds, often with quinone or iminoquinone functional groups, have been described to inhibit IDO1. It is possible that they inhibit IDO1 by redox-cycling with the reducing cofactors in the enzymatic assay solution. The reduction potentials of quinones given in Table 1 of ref 59 have been cited extensively to argue that no correlation exists between reduction potentials and IDO1 inhibitory activities of quinone compounds. Some values given in this table differ substantially from reduction potentials measured in aqueous solution at pH 7,⁶⁰ but the same conclusion was reached based on computational work.⁶¹ Strikingly, a similar absence of correlation between reduction potentials and redox-cycling activity was found in a screening specifically designed to

Table 1. Composition of Enzymatic Assay Solution with Recommended Concentrations (Modified from Ref 53)

class	reagent	concentration	comment
protein	IDO1	~40 nM	Prone to autoxidation; only ferrous form active; substrate inhibition by L-Trp.
	bovine catalase	50–150 nM	Removes H ₂ O ₂ by converting it to H ₂ O and O ₂ .
substrate	O ₂	max 270 μM	Saturation value at room temperature (8.6 mg/L).
	L-Trp	max 100 μM	Substrate inhibition at higher concentrations.
reducing agent	sodium ascorbate	20 mM	Reduces methylene blue; needed to keep IDO1 in ferrous state.
	methylene blue	10 μM	Redox-cycling compound; electron carrier; needed to keep IDO1 in ferrous state.
solvent	buffer	100 mM	Phosphate buffer; pH 6.5; no salt added.
	DMSO	max 5%	Solubilizes ligand; in cellular tests max 2%.
detergent	Triton X-100	0.01%	Reduces risk of ligand aggregation.
ligand	organic molecule	max 1 mM	

detect redox-cycling compounds,⁶² suggesting that other factors may play a role.

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) antioxidant assay⁶³ was used in one work on IDO1 inhibitors to investigate if the scaffold's activity on IDO1 was due to its redox-cycling properties.¹⁹ However, the test only detects reducing species, so the inactivity of the oxidized form of the scaffold was predictable. A more meaningful test would be if, under the employed enzymatic assay conditions, redox-cycling agents are able to inactivate IDO1 by oxidizing the available ascorbate/methylene blue resources. Hinting at this, it was shown that menadione (2-methyl-1,4-naphthoquinone) does not inhibit IDO1 when cytochrome P450 reductase/NADPH and cytochrome *b*₅ are used as reductants.⁵⁶ Redox-cycling compounds will not only affect the kynurenine pathway of Trp degradation but also interfere with many other cellular pathways, so their use for IDO1 inhibition should be avoided.

Chemical Reactivity and Promiscuous Behavior. Compound promiscuity due to aggregation,^{64–66} chemical reactivity, instability, or interference with assay detection methods frequently occurs among screening hits. Detection of these undesirable artifacts has traditionally been the task of experienced medicinal chemists in pharmaceutical companies. However, with the expansion of academic screening platforms, a growing number of promiscuous compounds are not recognized as such and end up in the scientific literature. This problem is receiving increasing attention,⁵² as reflected by a new section added to the author guidelines of the *Journal of Medicinal Chemistry* in 2015 (section 2.1.9 Interference Compounds). Tools to detect problematic compounds have been made publicly available by different companies and research groups.^{67–72}

In the case of IDO1, it has been found that the selenic compound ebselen inhibits the enzyme by binding to several of its cysteine residues.⁷³ In our own work, we observed that isothiazolinone derivatives probably inhibit IDO1 through covalent modification of nucleophilic amino acid side chains, since their steep dose-response curves (Hill coefficient of >1) strongly responded to the addition of a nucleophile to the

incubation medium.⁶¹ Cysteine reactivity has been identified as a major mechanism of promiscuity in different screens^{59,74,75} and can be predicted by the ALARM NMR cheminformatics filter.⁶⁹

Inhibition by aggregators^{64–66} is a frequent problem that can easily be reduced by addition of small amounts of nonionic detergent.⁷⁶ We observed that the presence of 0.01% of Triton X-100 in the incubation medium did not influence IDO1 activity and allowed us to detect aggregation-based inhibition, justifying its addition to our standard assay protocol.⁶¹ However, to our knowledge other published IDO1 inhibitors have not been tested in the presence of detergent.

Quantification of Enzymatic Reaction Product. The amount of L-Trp breakdown can be monitored most precisely by HPLC after stopping the enzymatic reaction by acidification and hydrolysis of NFK to kynurenine at 50 °C. In this way, kynurenine and L-Trp can be separated from other compounds and quantified individually.⁵³ Instead of this time-consuming technique (10–20 min per sample), in many experiments a direct spectroscopic detection without prior separation is used. The detection of either the primary enzymatic reaction product NFK (absorption maximum at 320 nm) or its hydrolyzed form, kynurenine (360 nm), is problematic because of the relatively short and unspecific wavelengths at which many other organic compounds absorb. Therefore, most commonly, kynurenine is reacted with *p*-dimethylaminobenzaldehyde (*p*DMAB), also called Ehrlich's reagent, to produce a compound with an absorption maximum at 480 nm.⁵³ However, since Ehrlich's reagent reacts with many common chemical functionalities such as ketones, aldehydes, hydrazines, and indoles, this technique is only reliable to compare samples that differ only in their amount of kynurenine. Particularly, the presence of different concentrations of small organic molecules or other Trp metabolites⁷⁷ can interfere with this detection method. Kynurenine contents can also be quantified using its weak fluorescence, with an excitation wavelength of 365 nm and an emission wavelength of 480 nm.⁷⁸ More specific quantification methods for NFK or kynurenine using fluorescence spectroscopy have been developed^{78–81} but not yet been widely adopted.

Substrate Inhibition. Substrate inhibition of IDO1 by L-Trp has been described as early as 1967⁵⁷ and was later explained by a second inhibitory L-Trp binding site.^{82–85} However, more recently the observed kinetics have convincingly been attributed to sequential ordered binding, first of O₂ and then of L-Trp, which is hindered at high L-Trp concentrations.^{86,87} These observations together with the fact that plasma concentration of L-Trp in healthy humans is about 65 μM¹⁸ argue against the use of L-Trp concentrations above 100 μM in the enzymatic assay. D-Trp has an about 100 times lower affinity for IDO1 than L-Trp and does not show substrate inhibition.^{82,86,87}

Inhibition Kinetics. For most IDO1 inhibitors, non-competitive or uncompetitive inhibition kinetics with respect to the substrate L-Trp were reported, although the compounds were assumed to bind to the same site. However, at least three factors complicate the measurement and interpretation of IDO1 inhibition kinetics.

First, L-Trp is not the only reaction substrate, and it has been shown that O₂ needs to bind to IDO1 before L-Trp.^{86,87} Therefore, if a compound is competitive with respect to oxygen, it is not competitive with respect to L-Trp. Here, the measurement of inhibition kinetics with respect to oxygen concentration could yield additional information. One of the

few inhibitors that have been confirmed by different groups to be competitive with respect to L-Trp is its analogue 1-methyl-L-tryptophan (L1MT),^{19,88–91} which apparently does not interfere with oxygen binding.

Second, experimental difficulties in determining inhibition kinetics arise from the observed substrate inhibition of IDO1 by L-Trp, which hampers the exact determination of V_{max} . Notably, the K_i values of the competitive hydroxyamidine compounds from Incyte were determined with the alternative substrate D-Trp that does not show substrate inhibition.³²

A third complication arises from the preferential binding of some inhibitors to the inactive ferric form of IDO1. For example, 4-phenylimidazole (4PI) was shown to have a 40 times higher affinity for ferric rabbit small intestine IDO1 than for its ferrous form.⁹² It has therefore been suggested that 4PI inhibits IDO1 by preventing its reductive activation, resulting in apparently noncompetitive inhibition kinetics,⁹² although it binds directly to the heme iron^{29,92} and occupies the presumed Trp binding site. Similar kinetics might, however, be obtained for unspecific compounds that interfere with the methylene blue/ascorbate reducing cofactors instead of binding to the IDO1 active site.

In summary, kinetic measurements with variable L-Trp concentrations do not yield direct information if a compound binds to the IDO1 active site or not.

Heme UV Absorption Spectra. UV absorption spectra of the characteristic heme peaks have been used to argue for a direct binding of compounds to the iron ion in the IDO1 active site.^{32,93,94} However, these peaks are sensitive to all structural changes occurring in its vicinity, not only to direct iron binding. For example, it has been shown that the binding of the selenic compound ebselen to cysteine residues of IDO1 shifts the Soret band of ferric IDO1 from 404 to 411 nm.⁷³

Cellular Assay. In a cellular context, IDO1 reductants are bound to be physiological and different from the ones used in the standard enzymatic assay. Here, different issues arise, such as off-target effects, the measurement of cell viability, and the quantification of the enzymatic reaction product from a complex mixture. If compounds display better cellular IC₅₀ values than their enzymatic counterparts, this should warrant caution about their mechanism of action because it could be due to off-target effects or the different reducing cofactors.

Off-Target Effects. Inside a cell, small organic molecules at micromolar concentrations may interact with many different targets. Two groups of proteins especially prone to be modulated by IDO1 inhibiting compounds are other heme proteins, such as for example cytochrome P450s, and other oxidoreductases. Because of their structural and functional similarity to IDO1, cross-reactivity against TDO and IDO2 should be investigated whenever possible. Another target potentially modulated by IDO1 ligands is the aryl hydrocarbon receptor (AhR) as a likely downstream mediator of kynurenines.¹¹ It has been reported that IDO1 activity is modulated by antioxidants both at the transcriptional level and posttranslationally by limiting the availability of heme.⁹⁵

Some quinones and iminoquinones display cellular IC₅₀ values much higher than their enzymatic IC₅₀ values^{31,59} and close to their LD₅₀ values, suggesting that the prevention of L-Trp to NFK oxidation could result from perturbing the cells' redox state instead of specific inhibition of IDO1. For other scaffolds like N-hydroxyamidines or 4-phenyl-1,2,3-triazoles it has been found that cellular IC₅₀ values were somewhat lower than the corresponding enzymatic values, but the ranking of

compounds by potency was roughly the same.^{32,94} In these cases the differences were attributed to different pH or different reducing cofactors.

Cell Viability. The measurement of cell viability is mandatory when reporting cellular IC₅₀ values because inhibition of Trp degradation could simply be an effect of cytotoxicity. Frequently, tetrazolium reduction assays with MTT or MTS are used for determining cell viability in the presence of different amounts of small organic molecules. This might not be the best choice, as these assays depend on the amount of reduced NAD(P)H in the cell, which may decrease in the presence of a redox-cycling compound. Alternative tests that have been used to validate cellular dose-response curves on IDO1 are the trypan blue exclusion assay,⁹⁶ the lactate dehydrogenase (LDH) assay,⁹⁷ the sulforhodamine B (SRB) assay,^{98,99} and an ATP assay.^{81,97}

Quantification of Enzymatic Reaction Products. Here, the same considerations as for the enzymatic assay are valid, but separation of Trp and NFK/kynurene from other compounds is even more important in the complex cellular environment. Particularly, the presence of other Trp metabolites has been shown to interfere with the detection of kynurene via Ehrlich's reagent.⁷⁷ Additionally, the measurement of L-Trp consumption should be considered more reliable than the measurement of NFK production, because oxidation of L-Trp is the rate-limiting step along this pathway, and downstream metabolism may be fast.

Structure-Based Drug Design. Two crystal structures of IDO1 published in 2006²⁹ opened the way for structure-based in silico design of IDO1 inhibitors. In the 4PI-bound X-ray structure (PDB accession code 2D0T, Figure 1A), the ligand is bound in a deep binding site with its phenyl ring inside a hydrophobic pocket (pocket A) and the imidazole coordinated to the heme iron. Another hydrophobic pocket at the entrance of the binding site (pocket B) is occupied by buffer molecules. The cyanide-bound structure (2D0U, not shown) differs from the 4PI-bound structure mainly in the hindered access to pocket A and illustrates the active site flexibility of IDO1. Recently, two new X-ray structures of IDO1 bound to the larger ligands 2 (Amg-1,⁴² PDB accession code 4PK5, Figure 1B) and a novel imidazothiazole inhibitor (4PK6, Figure 1C), which both occupy pocket A and pocket B, became available.³⁰ High side chain and backbone flexibility lead to different active site conformations, characterized by a larger A pocket and different shapes and sizes of the B pocket (Figure 1).

This flexibility poses a problem for IDO1 docking studies, where the receptor structure is usually held rigid. For example, 2 was predicted to bind at the entrance of the active site by docking to X-ray structure 2D0T,⁴² while X-ray crystallography later demonstrated a completely different binding mode, requiring a larger A pocket.³⁰ We observed that antifungal imidazoles such as miconazole only fit in the enlarged IDO1 active site as present after molecular dynamics simulations.⁶¹

A second issue for IDO1 docking studies is the presence of the heme iron in the active site, which can interact with ligands by forming covalent bonds. To treat this interaction, we developed a special Morse-like metal binding potential fitted to density functional theory calculations.¹⁰⁰ Most other docking studies on IDO1 used the GOLD docking algorithm, which takes heme binding into account via a knowledge-based potential.¹⁰¹ However, the strong sensitivity of IDO1 to the electronic nature of the iron-binding group is difficult to reliably describe in any

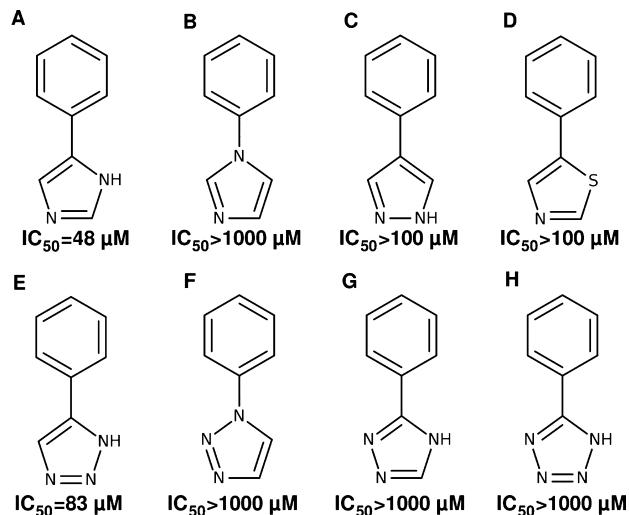


Figure 2. Bioisosteric replacements of the imidazole ring in 4PI (A). Enzymatic IC_{50} values from refs 59 and 94 are reported. (A) 4PI, (B) 1-phenylimidazole, (C) 4-phenylpyrazole, (D) 5-phenyl-1,3-thiazole, (E) 4-phenyl-1,2,3-triazole, (F) 1-phenyl-1,2,3-triazole, (G) 3-phenyl-1,2,4-triazole, and (H) 5-phenyltetrazole.

computational approach. An example of this sensitivity is highlighted in Figure 2. Bioisosteric replacements of the imidazole ring in 4PI by other nitrogen-containing five-membered rings lead to activity variations of several orders of magnitude.

In silico design has significantly contributed to the development of IDO1 inhibitors, but conflicting binding modes have been reported, for example, for L-Trp,^{31,42} 2-mercaptopbenzothiazole,^{31,102,103} and 4-phenyl-1,2,3-triazole,^{94,104} pointing out the problem of the validity of predicted binding modes. Caution should be used when interpreting docking results, taking into account that protein flexibility and heme interactions could be ill-described within the chosen approach. This is a bigger problem for completely new chemotypes compared to, for example, 4PI derivatives, which closely resemble an available cocrystallized ligand.

■ LEAD DISCOVERY FOR IDO1

IDO1 inhibitors known before the implication of this enzyme in tumoral immune resistance^{13,17,105} were of moderate micromolar activity, as outlined in a review in 2005.¹⁰⁶ Many of these were analogs of the substrate L-Trp^{36,88,107–110} or known heme binders such as 4PI⁹² and norharman.^{92,108,111,112} Over the past 10 years, new IDO1 inhibitor scaffolds have been discovered by natural product screening, structure-based drug design, or in the majority of cases by high throughput screening (HTS).

Natural-product-derived IDO1 inhibitors were frequently isolated from marine organisms.^{19,91,99,113–126} While providing good starting points in general for the development of new therapeutics, they also feature some drawbacks. The typical complexity and large molecular weight of natural products make it often difficult to derive druglike compounds. In the case of IDO1, a large number of natural product inhibitors contains problematic functional groups such as quinones. Frequently, only enzymatic assay results for one or a few compounds were reported, hindering the evaluation of their potential usefulness.

Structure-based design of new IDO1 inhibitors has so far been based on the 4PI-bound X-ray structure.^{31,61,93,94,98,103,127–129} Additionally, in many studies lead compounds were docked into

the 4PI-bound IDO1 structure to rationalize some features or structure–activity relationships.^{32,42,61,99,102,104,121,130,131}

HTS campaigns against IDO1 usually used variations of the common enzymatic inhibition assay protocol with ascorbate and methylene blue as reductants. However, a few alternative techniques such as cellular assays, yeast growth assays, and differential scanning fluorimetry were also applied and are outlined below. 245 000 compounds of Amgen's proprietary library were screened against IDO1 and IDO2, detecting kynurenine formation by fluorescence and L-Trp consumption by the proprietary Bridge-IT Trp assay (Medomics, LLC).⁴² Screening of the Prestwick Chemical Library of 1200 FDA-approved drugs and the Maybridge HitFinder Collection of 14 000 small molecules, detecting NFK through absorbance at 320 nm, was described in detail.⁶¹ Most of the hits were identified as false positives or unspecific inhibitors failing confirmatory tests. Using the newly developed proprietary probe NFK Green with an excitation wavelength of 400 nm and an emission wavelength of 510 nm, enzymatic and cellular screens of 87 000 compounds for IDO1 and TDO inhibition led to the discovery of 36 hits with nanomolar activity for IDO1 and 331 hits for TDO.⁸¹ Measuring IDO1 binding instead of IDO1 inhibition, the Zenobia Therapeutics Fragment Library 1 of 352 fragments was screened for IDO1 binding by differential scanning fluorimetry.¹⁰² A cellular mIDO2 inhibition screen of 640 FDA-approved compounds yielded 43 hits that were subsequently also tested on IDO1.⁹⁶ A yeast growth assay conducted with 2500 compounds from the NCI Diversity Set and from algal extracts identified one IDO1 inhibitor with a low micromolar K_i .¹³² More recently, a new yeast growth assay was developed and applied to 50 000 compounds of the ChemBridge DiverSet compound library. A low hit rate (0.2%) and a high confirmation rate (76%) were reported, but no chemical structures were disclosed.⁹⁷

■ PUBLISHED IDO1 INHIBITORS

In the following we classify published IDO1 inhibitors and give examples of their chemical structures in Figures 3–10. A radar chart is displayed for each compound, showing experimental data in the lower half and cheminformatics data in the upper half. Enzymatic and cellular ligand efficiencies (LE, given in (kcal/mol)/non-hydrogen-atom)¹³³ represent the experimental results. When no cellular assay data were reported, the cellular LE is set to zero. Among available cheminformatics filters we chose to report the ALARM NMR filter,⁶⁹ which predicts thiol reactivity of small molecules, the widely used “pan assay interference compounds” (PAINS) filter,⁷⁰ and the Lilly MedChem rules identifying reactive or promiscuous compounds⁷¹ as the most relevant and complementary filters (Figure 3A). For each compound, axes were ordered in a way to maximize the displayed surface. Although important parameters such as selectivity, more general “leadlikeness”, and ADME properties are missing in these charts, they give a graphic impression of the suitability of the hits. The larger is the red surface, the more efficient and reliable is the compound.

Tryptophan Analogs. Many of the first known IDO1 inhibitors were Trp or indole derivatives^{36,88,107–110} (Figure 3), the oldest one being 2,5-dihydro-L-phenylalanine (Figure 3B)¹⁰⁷ and the most widely used being 1-methyl-L-tryptophan (L1MT, Figure 3C).³⁶ L1MT passes all structural filters, but its moderate activity ($K_i = 19–53 \mu M$) has been used in the past as justification for publishing equally weak inhibitors.

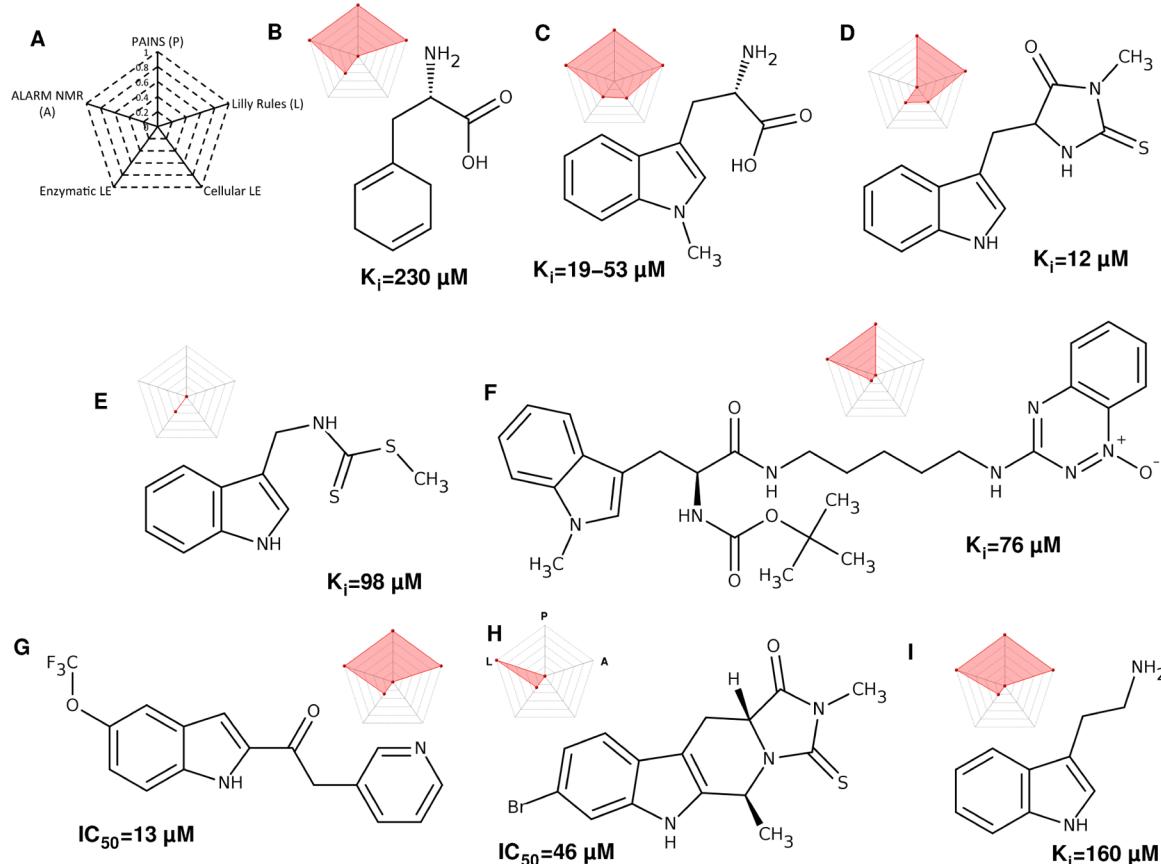


Figure 3. Tryptophan and indole analogs. Inhibition constants are given when available; otherwise enzymatic IC_{50} values are reported. (A) Radar chart for lead classification showing experimental results in the lower half and results from cheminformatics filters in the upper half (1 = passed, 0 = failed). Enzymatic ligand efficiency (LE), cellular LE (both in kcal/mol)/non-hydrogen-atom), Abbot ALARM filter (A),⁶⁹ PAINS filter (P),⁷⁰ and Lilly MedChem rules (L).⁷¹ When several experimental values or an activity range was available, LE values were calculated from the lowest value. When the ordering of the axes was changed to maximize the filled surface, this is indicated next to the respective chart. (B) 2S-Dihydro-L-phenylalanine,¹⁰⁷ (C) 1-methyl-L-tryptophan (L1MT),^{19,36,88-91} (D) MTH-trp or necrostatin-1,¹⁷ (E) brassinin,¹¹⁴ (F) L1MT/tirapazamine hybrid 1 from ref 90, (G) keto-indole 7m from ref 128, (H) tryptoline derivative 11c from ref 136, and (I) tryptamine.¹³⁷

In 2005, the indole derivative 5-((1*H*-indol-3-yl)methyl)-3-methyl-2-thioxoimidazolidin-4-one (Figure 3D) was reported as IDO1 inhibitor ($K_i = 12 \mu\text{M}$) under the name MTH-trp.¹⁷ In the same year, this compound was independently shown to be a necroptosis inhibitor (cellular $IC_{50} = 0.5 \mu\text{M}$)¹³⁴ and widely used as such under the name necrostatin 1. In 2013 only, the dual role of this compound was highlighted and the advice was issued to use more selective compounds for studying the two pathways separately.¹³⁵

The brassinin-derived dithiocarbamates (Figure 3E),¹¹⁴ for which only enzymatic assay results are available, fail all filters. More recently developed Trp derivatives with micromolar IDO1-inhibitory activity include 1-methyltryptophantirapazamine compounds as hypoxia-targeting IDO1 inhibitors (Figure 3F),⁹⁰ keto indoles from a virtual screen (Figure 3G),^{93,128} tryptoline derivatives (Figure 3H),¹³⁶ and tryptamine derivatives (Figure 3I).¹³⁷

As of today, no Trp analog reached submicromolar activities, probably because Trp itself shows only a moderate affinity to IDO1 ($K_d = 290-320 \mu\text{M}$).^{29,138} A better approach for developing IDO1 inhibitors would be to mimic the transition state of the enzymatic reaction, which needs to take the iron-bound dioxygen into account.

Inhibitor Scaffolds with Quinone/Iminoquinone Motif.

Compounds with quinone or iminoquinone functionalities

(Figure 4) display high activities in enzymatic IDO1 inhibition assays.^{31,59,82,91,99,113,115-117,120,121,123-125,139-143} Interestingly, for TDO already in 1961 the redox-active compounds catechol, hydroquinone, *p*-quinone, L-dihydroxyphenylalanine, and L-epinephrine were described as inhibitors.¹⁴⁴ For IDO1, the activity of menadione (Figure 4C) and related quinone compounds was first reported in the patent literature in 2006.¹³⁹ Quinones could either inhibit IDO1 by specific interaction with the enzyme active site or by redox-cycling with the reducing cofactors^{56,62} or by chemical reactivity toward nucleophilic amino acid side chains.^{59,69,74,75,145}

Besides enzymatic IDO1 inhibition results provided in all references, in some works also cellular inhibition results were reported.^{31,59,91,99,120,121} While in one article describing quinone inhibitors, activities reduced by 2–3 orders of magnitude were reported in cellular assays as compared to enzymatic assays,⁵⁹ in later works similar activities were found in both contexts. In vivo data for menadione (Figure 4C)¹³⁹ administered to a mouse melanoma model showed reduced tumor volume and IDO1-mediated efficacy.⁵⁹ Some benzofuranquinones (Figure 4J) were shown not to damage microtubules and the actin cytoskeleton, not to generate measurable levels of oxidative stress, and not to induce cytotoxicity,¹²⁴ at variance with β -lapachone (Figure 4H).⁹⁹

While the exact mode of action of the structurally highly diverse quinone compounds remains an open question, they fail

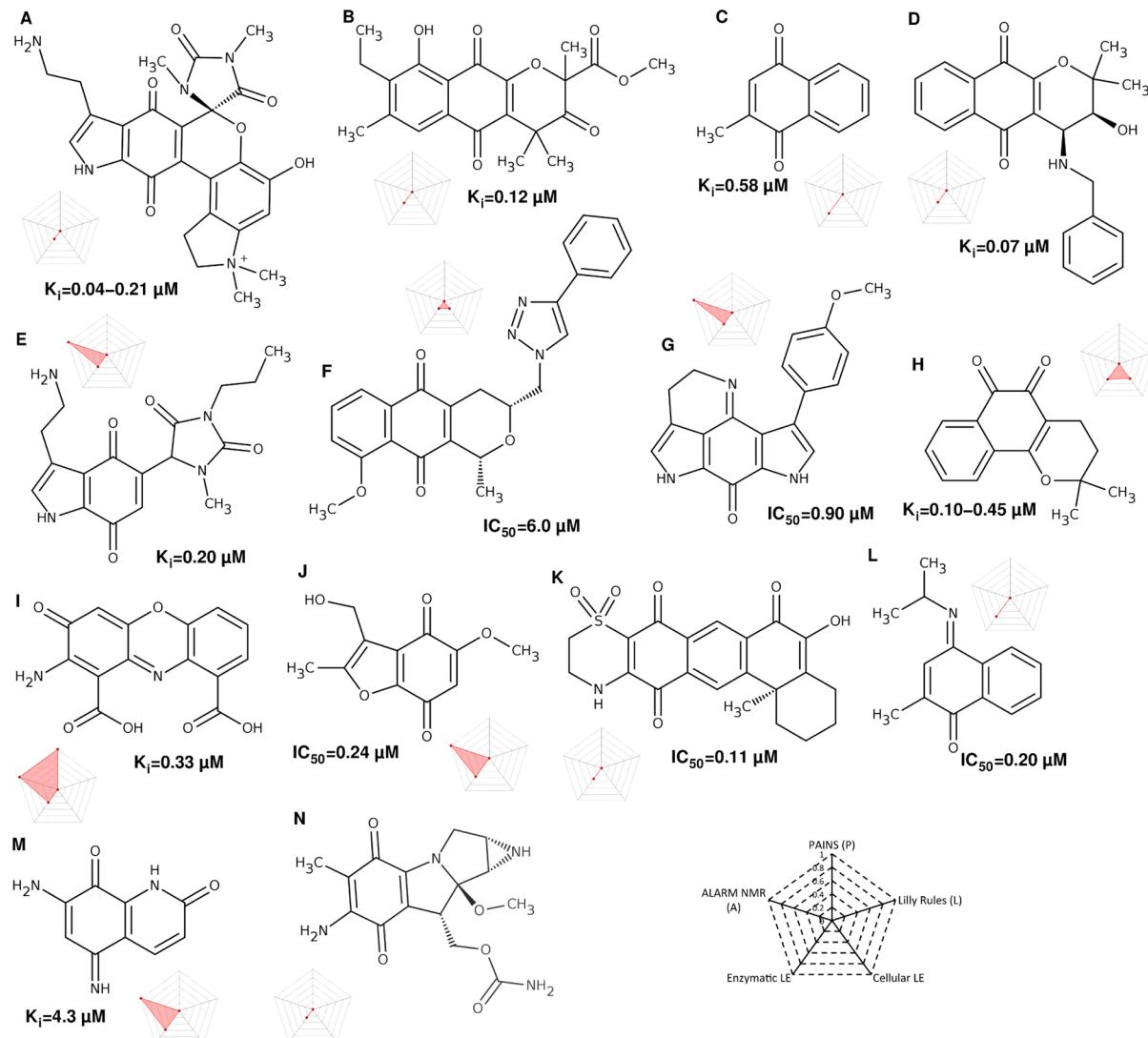


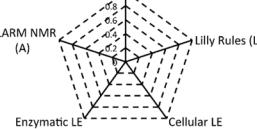
Figure 4. Inhibitor scaffolds with quinone or iminoquinone function. Inhibition constants are given when available; otherwise enzymatic IC_{50} values are reported. Details for the radar charts of lead classification are given in Figure 3A. (A) Exiguamine A,^{113,116} (B) annulin B,^{115,139} (C) menadione,^{59,139} (D) pyranonaphthoquinone 36 from ref 59, (E) indolequinone 22 from ref 116, (F) pyranonaphthoquinone 8 from ref 120, (G) pyrroloiminoquinone 11b from ref 121, (H) β -lapachone,^{99,124,141} (I) cinnabarinic acid,^{117,123} (J) benzofuranquinone 5a from ref 124, (K) xestosaprol O analog 3 from ref 125, (L) 4-iminonaphthalen-1-one derivative 55 from ref 31, (M) NSC111041 from the National Cancer Institute,¹⁴¹ and (N) mitomycin C.^{82,141}

most filters for promiscuous compounds and it seems evident that they do not represent a suitable starting point for developing highly selective IDO1 inhibitors.

Other Natural-Product Inhibitors. The first known natural-product inhibitors of IDO1 were the moderately active norharman or β -carboline (Figure 5A) derivatives.^{92,108,111,112} Other natural-product inhibitors include benzomalvin E (Figure 5B),¹¹⁸ halicloic acids A and B (Figure 5C),¹¹⁹ and thielin derivatives (Figure 5D),¹²² which all showed activity in an IDO1 enzymatic assay, but no further analysis was carried out. Tryptanthrin derivatives (Figure 5E) showed activity both in enzymatic and in cellular IDO1 inhibition assays, in a T-cell proliferation assay, in a surface plasmon resonance binding assay, and in Lewis lung cancer tumor-bearing mice.¹⁹ The phytochemical galanal (Figure 5F) showed activity in enzymatic and in cellular IDO1 inhibition assays, but cell viability was not reported.¹²⁶

Scaffolds with a Single Aromatic Ring. Most inhibitor scaffolds for IDO1 contain at least two aromatic rings, exceptions

being the scaffolds shown in Figure 6, which in some cases seem to compensate small size by chemical reactivity. O-Benzylhydroxylamine (Figure 6A) and derivatives with activities in the single-digit micromolar range were described in a patent in 2009,¹²⁷ reporting both enzymatic and cellular assay results. Phenylhydrazine (Figure 6B) was discovered by fragment screening and reported to be a potent enzymatic and cellular IDO1 inhibitor.¹⁰² However, its selectivity for IDO1 over other heme proteins and its reversibility remain to be investigated. The scaffolds benzyl mercaptan (Figure 6C)¹⁴⁶ and S-benzylisothiourea (Figure 6D)¹⁴⁶ were tested in enzymatic and in cellular assays. However, cell viability was not reported, and the compounds fail most cheminformatics filters for their reactivity. Modification of the earlier described 2(3*H*)-benzothiazolethione scaffold³¹ based on its docked conformation yielded a series of *N*-phenylthiosemicarbazides, the best one displaying an enzymatic IC_{50} value of 1.2 μM (Figure 6E).¹⁰³ The structurally related compounds shown in Figure 6F¹¹⁴ and Figure 6G¹²⁹ show somewhat weaker enzymatic activities.



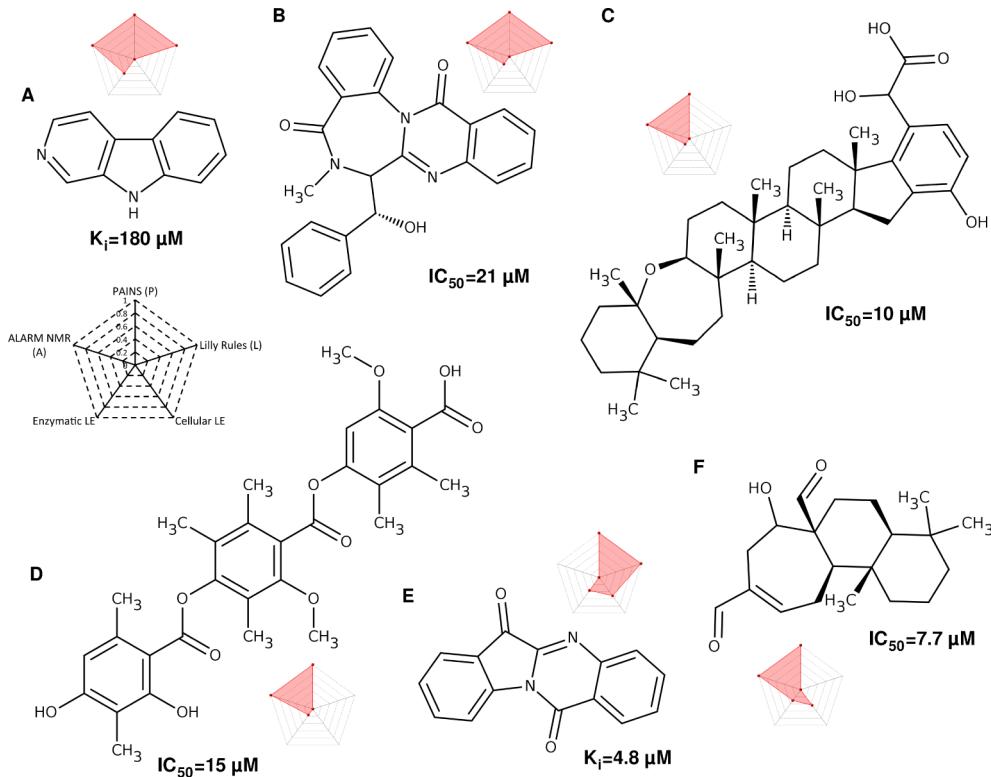


Figure 5. Other natural product inhibitors. Inhibition constants are given when available; otherwise enzymatic IC_{50} values are reported. Details for the radar charts of lead classification are given in Figure 3A. (A) Norharman/β-carboline,^{92,108,111,112} (B) benzomalvin E,¹¹⁸ (C) halicloic acid A,¹¹⁹ (D) thielavin F,¹²² (E) tryptanthrin,¹⁹ and (F) galanal.¹²⁶

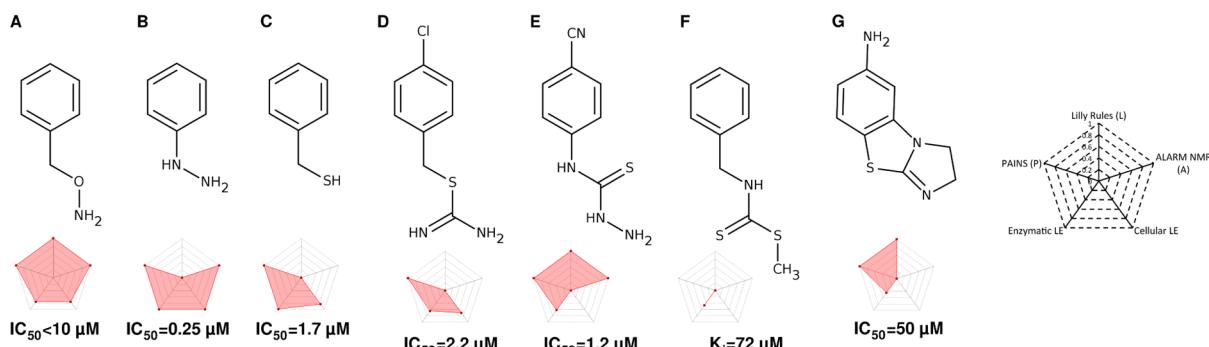


Figure 6. Inhibitors with a single aromatic ring. Inhibition constants are given when available; otherwise enzymatic IC_{50} values are reported. Details for the radar charts of lead classification are given in Figure 3A. (A) O-Benzylhydroxylamine,¹²⁷ (B) phenylhydrazine,¹⁰² (C) benzyl mercaptan,¹⁴⁶ (D) S-benzylisothiourea derivative 3d from ref 146, (E) thiosemicarbazide 14 from ref 103, (F) dithiocarbamate derivative 8 from ref 114, and (G) AC12308 from the Maybridge Screening Collection.¹²⁹

Because of their different chemical functionalities, these compounds show different suitability profiles, spanning from an almost filled radar chart (Figure 6A) to an almost empty one (Figure 6F). However, a common issue for these small compounds will be their selectivity.

Imidazoles. 4-Phenylimidazole (4PI, Figure 7A), a known heme binder, was described early on as IDO1 inhibitor⁹² and gained additional attention due to its cocrystallization with IDO1 in 2006.²⁹ 4PI derivatives up to 10-fold more potent than the parent compound were designed by a structure-based approach (Figure 7B).⁹⁸ Fungistatic drugs of the imidazole type such as miconazole and econazole (Figure 7C) were discovered in two independent screens to be active against IDO1, while similar 1,2,4-triazole drugs such as fluconazole were completely inactive.^{61,96} Structure-based modifications of the 1-substituted

imidazole antifungal scaffold led to more soluble but less potent compounds (Figure 7D).⁶¹ NewLink Genetics developed further 4-substituted phenylimidazoles with nanomolar potency but a low LE because they featured a long extension into the B pocket (Figure 7E).¹⁴⁷ Fusion of the two aromatic rings of 4PI by an aliphatic carbon atom (Figure 7F), however, enhanced potency with less impact on efficiency.¹⁴⁸ Replacement of the imidazole motif in the fused compound by other heterocycles such as 1,2,3-triazoles led to much less active compounds, while the orientation of the fused imidazole ring seemed to be less important (Figure 7G).¹⁴⁹

In summary, the phenylimidazole scaffold provides a promising starting point for the development of IDO1 inhibitors, as its binding mode to the active site is known through X-ray crystallography,²⁹ it passes all structural filters,

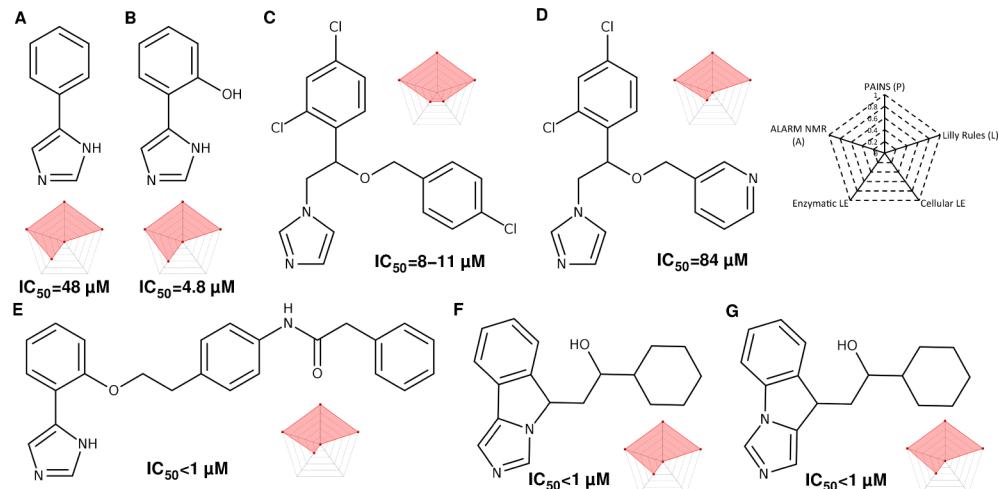


Figure 7. Inhibitors with imidazole scaffold and their reported enzymatic IC_{50} values. Details for the radar charts of lead classification are given in Figure 3A. (A) 4-Phenylimidazole (4PI),^{92,98} (B) 4PI derivative 1 from ref 98,⁶¹ (C) econazole,^{61,96} (D) derivative of fungistatic imidazoles 6b from ref 61, (E) 4PI derivative 1102 from ref 147,¹ (F) fused imidazole 1304 from ref 148,¹ and (G) fused imidazole 70 from ref 149.

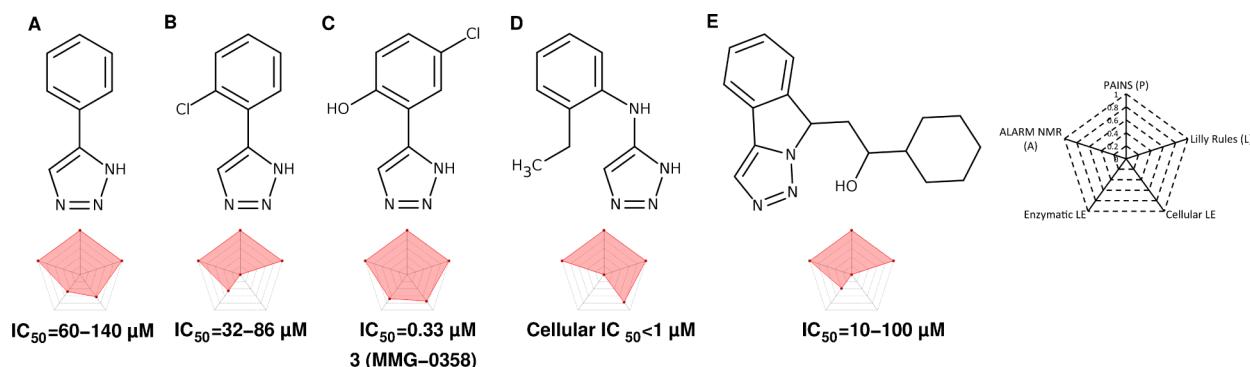


Figure 8. Inhibitors with 1,2,3-triazole scaffold and their reported enzymatic IC_{50} values. Details for the radar charts of lead classification are given in Figure 3A. (A) 4-Phenyl-1,2,3-triazole,^{31,94,104} (B) compound 8 from ref 104,¹ (C) compound 3,⁹⁴ (D) compound 1 from ref 51,¹ and (E) fused triazole 66 from ref 149.

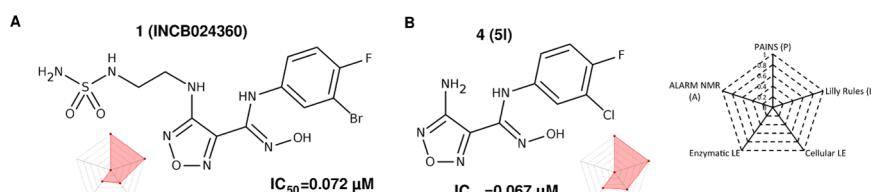


Figure 9. N-Hydroxyamidine inhibitors from Incyte and their reported enzymatic IC_{50} values. Details for the radar charts of lead classification are given in Figure 3A. (A) Epacadostat (1),¹⁵¹ (B) compound 4.³²

rational modifications have been shown to be feasible, and sensible structure–activity relationships are observed. The fact that 4PI and the fungistatic imidazoles inhibit various heme enzymes suggests that specificity for IDO1 needs to be achieved through optimized molecular recognition by the B pocket.

1,2,3-Triazoles. 4-Aryl-1,2,3-triazoles were discovered in 2010, with the parent compound displaying an enzymatic IC_{50} value of 60 μM and passing all structural filters (Figure 8A).³¹ In one subsequent work, only derivatives with similar or lower potency were reported (Figure 8B).¹⁰⁴ However, the rationally designed triazole **3** (MMG-0358, Figure 8C)⁹⁴ showed nanomolar activities in both enzymatic and cellular assays. Additionally, it demonstrated low cellular toxicity and a high selectivity for IDO1 over TDO.⁹⁴ Nanomolar cellular IC_{50} values were also reported for

the *N*-phenyl-1,2,3-triazol-4-amine compounds from Vertex (Figure 8D).⁵¹ As mentioned above, bioisosteric replacement of imidazole by 1,2,3-triazole in the fused compounds from NewLink Genetics led to only moderately active compounds (Figure 8E).¹⁴⁹

In conclusion, the 1,2,3-triazole scaffold provides an interesting alternative to the imidazole scaffold, as it could exhibit better specificity with respect to other heme proteins.

N-Hydroxyamidines. HTS of Incyte's corporate collection led to the discovery of *N*-hydroxyamidines as potent, reversible, competitive IDO1 inhibitors (Figure 9).³² The most potent compound of this series, compound **4** (5I, Figure 9B),³² displayed enzymatic and cellular IC_{50} values of 67 and 19 nM, suppressed kynurenine generation *in vivo*, and inhibited melanoma growth in a mouse model. Modeling of the binding of **4** to IDO1 assumed that the oxygen of the hydroxyamide

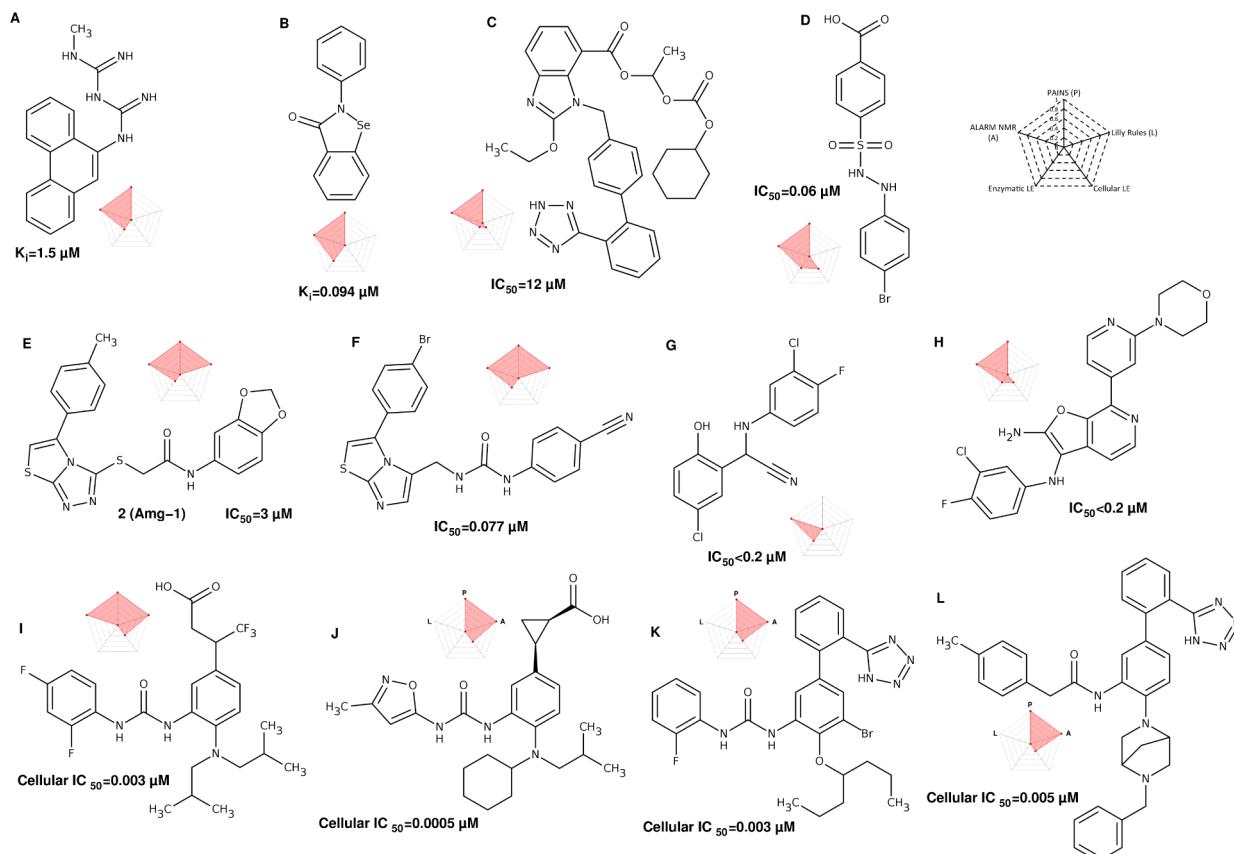


Figure 10. Other inhibitors with published activity data. Details for the radar charts of lead classification are given in Figure 3A. (A) NSC401366 from the NCI Diversity Set,¹³² (B) ebselen,⁷³ (C) candesartan cilexetil,¹³⁰ (D) benzenesulfonyl hydrazide 3i from ref 131, (E) compound 2,⁴² (F) imidazothiazole 17g from ref 30, (G) aminonitrile 30 from ref 47, (H) compound 154 from ref 48, (I) 2-aminophenylurea 13 from ref 43, (J) 2-aminophenylurea 129 from ref 44, (K) compound 287 from ref 45, (L) compound 80 from ref 46.

binds to the heme iron and forms a hydrogen bond with the aniline nitrogen. The phenyl ring was placed inside the A pocket, producing a tight fit, while the amino substituent on the furazan ring could form a hydrogen bond to the propanoic acid group on the heme ring.³² Compound 4 was used extensively as reference compound by other groups and was erroneously sold under the name of the clinical candidate 1 by several chemical vendors. The correct structure of 1 (epacadostat, CAS no. 1204669-58-8, Figure 9A) was recently disclosed and belongs to a further optimized series of *N*-hydroxyamidines with extension to the B-pocket.^{150,151} The scaffold fails the ALARM NMR filter because of its furazan group (Figure 9). Epacadostat was shown to inhibit IDO1 in enzymatic ($IC_{50} = 72$ nM) and in cellular tests ($IC_{50} = 7.1$ nM), to suppress Trp catabolism in vivo, to impede tumor growth, and not to inhibit IDO2, TDO, tryptophan transport, or a panel of 50 other proteins.^{33,152} Its proposed binding mode, analogous to the one of 4, is outlined in Figure 11.

Other Scaffolds. One IDO1 inhibitor from the NCI Diversity Set with a K_i of $1.5\ \mu M$ (Figure 10A) was reported in 2006.¹³² The selenic antiinflammatory antioxidant ebselen (Figure 10B) reportedly inhibits IDO1 by binding to several of its cysteine residues.⁷³ The prodrug candesartan cilexetil (Figure 10C) was found to inhibit IDO1 with an enzymatic IC_{50} value of $12\ \mu M$.¹³⁰ The active form of this angiotensin II receptor antagonist, obtained after ester hydrolysis, was reported to be a much weaker inhibitor. Replacing the ester by different amides led to 10-fold increase in enzymatic activity

but did not improve the cellular IC_{50} value of $2.6\ \mu M$ of candesartan cilexetil.¹³⁰ A series of benzenesulfonyl hydrazides were tested in enzymatic and cellular IDO1 inhibition assays, displaying nanomolar IC_{50} values in both tests (Figure 10D).¹³¹ However, cell viability was not tested.

Amgen reported compound 2 (Figure 10E) to inhibit IDO1 with an IC_{50} value of $3\ \mu M$, and its selectivity for IDO1 over IDO2 and TDO was demonstrated.⁴² This compound was later cocrystallized with IDO1 (Figure 1B) and used for rational compound optimization, which led to the discovery of imidazothiazole derivatives occupying both pocket A and pocket B (Figure 1C).³⁰ Nanomolar enzymatic inhibition activities were obtained with a urea linker, the best compound showing an enzymatic IC_{50} value of 77 nM (Figure 10F).

Recently, the pharma company Curadev reported aminonitriles as potent IDO1 inhibitors (Figure 10G).⁴⁷ However, most of these fail the PAINS filter as phenolic Mannich bases and the Lilly MedChem filter because of their cyanomethyl-amine functionality. In a second report, extensive enzymatic, cellular, and in vivo data were provided for a different scaffold, diamino substituted furopyridines (Figure 10H).⁴⁸ Here, the tolerance of bulky substituents in both 3 and 7 positions makes it difficult to imagine how this scaffold could fit into the IDO1 active site.

Bristol-Myers Squibb described a 2-aminophenylurea scaffold and related compounds for IDO1 inhibition yielding low nanomolar to picomolar cellular IC_{50} values (Figure 10I–L).^{43–46} These are the most potent reported compounds to date,

but little data are available about their specificity and mode of action. Also in this case, their three-dimensional structure does

Table 2. Recommendations To Avoid Promiscuous or Unspecific Inhibition of IDO1

experiment	recommendation
general	<ul style="list-style-type: none"> Use cheminformatics filters and medicinal chemistry knowledge to detect promiscuous compounds. Add nonionic detergent to assay solutions to reduce risk of aggregation. Avoid redox-cycling lead compounds. Test compound solubility and report DMSO content. Check for cross-reactivity against IDO2 and TDO. Use L-Trp concentration not exceeding 100 μM to reduce substrate inhibition.
enzymatic assay	<ul style="list-style-type: none"> Report Hill coefficients for all dose-response curves. If deviating from 1, investigate mode of action by additional experiments. Test for irreversible and cofactor-dependent inhibition. Test interference of compounds with assay readout.
cellular assay	<ul style="list-style-type: none"> Report LD₅₀ values. Cell viability test should be orthogonal to IDO1 assay. Report correlation between enzymatic and cellular data. In the case of low correlation, investigate mode of action by additional experiments. Test interference of compounds with assay readout. Take influence of oxygen substrate into account.
inhibition kinetics	<ul style="list-style-type: none"> Take influence of substrate inhibition by L-Trp into account.

not seem to be able to fill the IDO1 active site without clashing with surrounding protein residues. In addition to these patented compounds, Bristol-Myers Squibb acquired the company Flexus Biosciences in February 2015 to develop their preclinical IDO1 inhibitor F001287 (structure not disclosed), targeted for IND filing in the second half of 2015.¹⁵³

CONCLUSIONS

Numerous different chemical series of IDO1 inhibitors have been described. However, many scaffolds have never been used after their initial report, probably because of unconfirmed activity and/or specificity in follow-up studies. Here, our main objective is to raise awareness that a compound inhibiting IDO1 function *in vitro* is not necessarily an IDO1 inhibitor and that additional tests are necessary to increase the likelihood of successful translation of *in vitro* to *in vivo* activity. To accelerate future progress, recommendations to avoid common problems are given in Table 2. Besides general suggestions on how to reduce the risk of promiscuous or unspecific inhibition, which are often disregarded in the literature reporting IDO1 inhibitors, also more specific aspects of IDO1, such as substrate inhibition and sensitivity to redox-cycling compounds, are accounted for in this table. A selection of viable IDO1 inhibitors together with their presumed binding modes to the IDO1 active site are depicted in Figure 11.

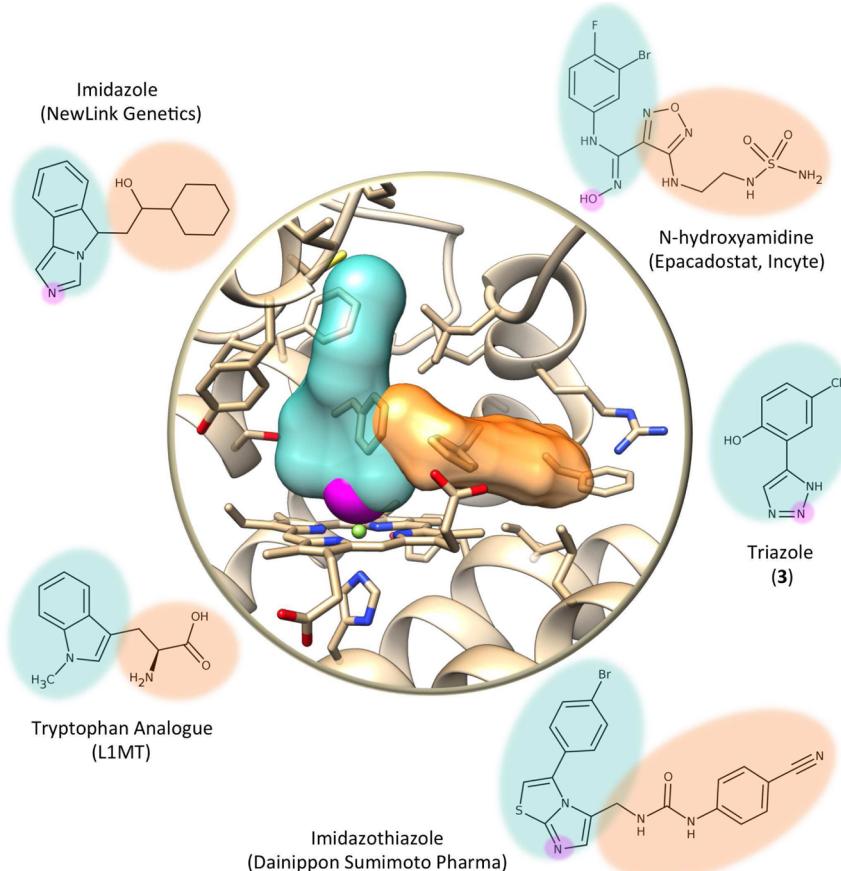


Figure 11. Viable IDO1 inhibitors. The IDO1 active site (PDB code 4PK5) is depicted with pocket A in turquoise, pocket B in orange, and the iron binding site in magenta. Around the active site, different inhibitors are shown, color-coded by their presumed binding mode and labeled with their scaffold type, common name, and developing company when available.

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Notes

The authors declare no competing financial interest.

Biographies

Ute F. Röhrig studied chemistry at the University of Hamburg and at the Ludwig-Maximilians-University in Munich with a scholarship from the German National Academic Foundation. In 2004 she obtained a Ph.D. in Computational Chemistry from the Swiss Federal Institute of Technology (ETH) in Zurich for her work on hybrid quantum/classical simulations of the photoreceptor rhodopsin under the guidance of Ursula Röthlisberger. After a postdoc at the Enrico Fermi Research Centre in Rome with Roberto Petronzio and Michele Parrinello, Ute F. Röhrig joined the Molecular Modeling Group at the SIB Swiss Institute of Bioinformatics in 2006. Her research interests include computational modeling and *in silico* drug design with experimental validation, especially using hybrid quantum/classical methods.

Somi Reddy Majjigapu was educated in India, receiving a Master's degree from Osmania University and a Ph.D. in Organic Chemistry from the Indian Institute of Chemical Technology, Hyderabad, under the direction of K. Rama Rao. During his doctoral studies, he was involved in the synthesis of bioactive natural products such as simplactone B and substituted piperidines, and he also developed new methodologies for organic synthesis. For postdoctoral training he moved to Cambridge Major Laboratories, USA, in 2008. In 2009, Somi Reddy Majjigapu joined the Swiss Federal Institute of Technology Lausanne (EPFL) and in 2010 also the SIB Swiss Institute of Bioinformatics, where he is working on the synthesis of antitumor agents and IDO1 inhibitors under the supervision of Pierre Vogel and Olivier Michielin.

Pierre Vogel received a Ph.D. in 1969 from the University of Lausanne, Switzerland, for his work with Horst Prinzbach. After working with Martin Saunders at Yale University and Pierre Crabbé at the research laboratory of Syntex in Mexico City, he returned to Lausanne where he was promoted to Full Professor in 1977. He obtained the Novartis Lectureship in 2003 and was elected Boehringer-Ingelheim Distinguished Lecturer in 2005. Since 2001, Pierre Vogel holds the Chair of the Laboratory of Glycochemistry and of Asymmetric Synthesis of the Swiss Federal Institute of Technology Lausanne (EPFL). He published three books and coauthored more than 550 publications in the fields of physical organic chemistry, organic and organometallic synthesis, catalysis, glycochemistry, and bioorganic and medicinal chemistry.

Vincent Zoete obtained an Engineer's degree in Chemistry from the ENSCL in 1995, a Master's degree in Drug Design in 1996, and a Ph.D. in Organic Chemistry from the University of Lille in 1999. He did his postdoctoral training in molecular modeling in the groups of Martin Karplus in Strasbourg and of Markus Meuwly in Basel. In 2004 he joined the Molecular Modeling Group of the Swiss Institute of Bioinformatics in Lausanne, where he currently is Associate Group Leader. Additionally, he is the scientific director of the Protein Modeling Facility of the University of Lausanne. His research interests include the development and application of computer-aided approaches for protein engineering and drug design. He contributed to the development of tools like SwissDock, SwissParam, SwissSide chain, SwissTargetPrediction, and SwissBioisostere.

Olivier Michielin is a medical oncologist specialized in melanoma and a bioinformatician involved in the rational design of new melanoma therapies. He obtained an Engineer's degree in Physics from the Swiss Federal Institute of Technology Lausanne (EPFL) in 1991, followed by a Medical degree from the University of Lausanne in 1997. In 2001, he obtained a M.D.-Ph.D. degree under the supervision of Martin Karplus for his work on protein structure/function relationships. Olivier Michielin started his own research group at the Swiss Institute of Bioinformatics in 2002. He is a member of the Ludwig Institute for Cancer Research and Associate Professor of Translational Oncology at the Lausanne University Hospital, where he has led the melanoma program and the outpatient clinic since 2007.

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ABBREVIATIONS USED

4PI, 4-phenylimidazole; HTS, high throughput screening; IDO1, indoleamine 2,3-dioxygenase 1; LE, ligand efficiency; L1MT, 1-methyl-L-tryptophan; NFK, N-formyl kynurenone; PAINS, pan assay interference compounds; TDO, tryptophan 2,3-dioxygenase; Trp, tryptophan

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