Inhibitors of the Kynurenine Pathway

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Abstract A central role in the immune escape of tumors has been attributed to the kynurenine pathway of tryptophan metabolism, leading to the depletion of tryptophan and the production of different bioactive metabolites. The first and rate-limiting step in this pathway is catalyzed by the phylogenetically unrelated enzymes indoleamine 2,3-dioxygenase 1 (IDO1) and tryptophan 2,3-dioxygenase (TDO), which have both been shown to be expressed in different cancers. Intense efforts in academia and in pharmaceutical companies to develop novel inhibitor scaffolds yielded a few compounds currently undergoing clinical trials. Here, we review the most significant compounds in the field and discuss potential issues in the development of kynurenine pathway inhibitors for cancer therapy.

Keywords Immuno-oncology, Indoleamine 2,3-dioxygenase, Kynurenine pathway, Tryptophan 2,3-dioxygenase, Tryptophan metabolism

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1 Introduction

Cancer immunotherapy is a rapidly evolving field [1], which has generated much excitement among scientists and cancer patients over the last years because of the efficacy of immune checkpoint inhibitors [2], chimeric antigen receptor T-cell therapy [3], and adoptive cell therapy with tumor infiltrating lymphocytes [4]. These promising new therapies, as well as more traditional approaches such as vaccination, chemotherapy, or radiotherapy could benefit from the combination with a strategy to overcome tumor-induced immunosuppression [5–7]. A central role in immune escape is played by the kynurenine pathway of L-tryptophan (L-Trp) metabolism (Fig. 1) [8–11]. This pathway, which has been estimated to be responsible for more than 99% of L-Trp degradation in humans [12, 13], leads to the depletion of L-Trp and to the production of kynurenine metabolites and causes local immunosuppression [14–17]. The pathway is not only involved in the immune system, for example, in establishing immune tolerance versus the fetus during pregnancy [18] and in the response to infections [19], but it also influences the nervous system and plays a major role in neurological and mental disorders [20, 21]. The first and rate-limiting step in the kynurenine pathway is the oxidation of L-Trp to N-formylkynurenine (NFK, Fig. 1). This reaction is catalyzed by two evolutionarily unrelated heme enzymes, tryptophan 2,3-dioxygenase (TDO, EC 1.13.11.11) and indoleamine 2,3-dioxygenase 1 (IDO1, EC 1.13.11.52). TDO is a homotetrameric enzyme, upregulated by glucocorticoid hormones and the availability of L-Trp, constitutively expressed in the liver, and responsible for regulating systemic L-Trp levels [16]. IDO1 on the other hand is a monomeric enzyme induced by interferon gamma and expressed in different cell types and tissues [16, 22]. IDO1 has first been implicated in tumoral immune resistance in 2003 [23], a discovery that initiated a very active field of drug

Fig. 1 Kynurenine pathway

development. The implication of TDO in similar mechanisms has been described more recently [24, 25]. The role of the IDO1 paralogue indoleamine 2,3-dioxygenase 2 (IDO2) [26–28] remains less clear due to ambiguous experimental findings. IDO2 shows a very low affinity for L-Trp and a low turnover rate [29]. Different issues have hampered biological studies of L-Trp metabolism, such as: (1) difficulties in obtaining gene knockout mice lacking both IDO1 and IDO2 [30], (2) antibody reagents against murine IDO1 lacking specificity [31, 32], and (3) the widespread use of high concentrations of the very unspecific IDO1 inhibitor 1-methyl-tryptophan (1MT), which has been shown to additionally induce IDO1 [24] and to be contaminated with L-Trp [33].

Here, we concentrate on the description of small molecule inhibitors of the first step of L-Trp metabolism, namely of IDO1, TDO, and IDO2, as these are the main targets for cancer therapy.

2 Indoleamine 2,3-Dioxygenase 1 Inhibitors

IDO1 (EC 1.13.11.52) is the most well-established target in the kynurenine pathway for cancer immunotherapy [22]. It has been shown to be expressed by tumor cells in order to escape a potentially effective immune response [14, 23, 34, 35], and high IDO1 expression is associated with poor prognosis in different cancer types [36]. In vitro and in vivo studies demonstrate that administration of an IDO1 inhibitor improves the efficacy of therapeutic vaccination, chemotherapy, or radiation therapy [23, 37–40].

IDO1 is a heme enzyme that catalyzes the oxidation of L-Trp to NFK by the incorporation of molecular oxygen and the cleavage of the pyrrole ring of the substrate. The crystal structures of human IDO1 [41–43] show one binding pocket in the distal heme site (Pocket A, Fig. 2a), connected to a second pocket towards the entrance of the active site (Pocket B, Fig. 2a) [44]. The “height” of the A pocket varies among different X-ray structures, as evident from Fig. 2a–d.

Intense efforts in academia and in the pharmaceutical industry to develop IDO1 inhibitors led to at least three compounds in clinical trials so far. The most advanced compound, at the time of writing, was the hydroxyamidinone **18** (INCB024360, epacadostat) [38, 45–47] developed by Incyte Corporation, which had been tested in combination with different other cancer therapies and partners in clinical trials up to phase III. A second compound in phase I clinical studies at the time of writing is the imidazole GDC-0919 (formerly known as NLG919 or RG6078, structure not disclosed) [48, 49] discovered by NewLink Genetics and developed together with Genentech. iTeos Therapeutics [50–53] in partnership with Pfizer initiated a phase I study with compound PF-06840003 (undisclosed structure) for patients with malignant gliomas (NCT02764151).

In addition to academic groups, the pharma companies Amgen [54], Bristol-Myers Squibb [55–58], Curadev [59–61] (partnering with Roche), Dainippon Sumitomo Pharma Corporation [42], Flexus Biosciences [62–65] (acquired by Bristol-Myers Squibb in 2015), Iomet Pharma [66–70] (acquired by Merck & Co. in 2016), Merck Group [71], Redx Pharma [72, 73], and Vertex Pharmaceuticals [74] have all reported work on the development of IDO1 inhibitors.

IDO1 inhibitory properties have been attributed to thousands of compounds, but many of them may act through unspecific mechanisms [75]. Here, we limit ourselves to discuss the most relevant and novel scaffolds. A complete review of IDO1 inhibitors described before February 2015 can be found in a recent review [75]. In the following figures, ligands are shown in their putative binding conformation to IDO1 according to Fig. 2 whenever possible, with the heme-iron facing group in the lower

Fig. 2 X-ray structures of indoleamine 2,3-dioxygenase 1 (IDO1) [41–43]. 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), and *orange* (iron). Hydrogen bonds are shown as *thin orange lines*. (a) Structure 2D0T bound to 4PI (7) and two molecules of CHES buffer (displayed in stick representation) [41]. The A pocket above the heme distal site and the B pocket towards the entrance of the active site [44] are highlighted. (b) Structure 4PK5 bound to compound 38 [42]. (c) Low resolution (3.45 Å) structure 4PK6 bound to an imidazothiazole derivative [42]. (d) Structure 5EK4 bound to compound 15, an analog of GDC-0919 [43]

left, the A-pocket binding group in the upper left, and the B-pocket binding group extending to the right.

2.1 1-Methyl-Tryptophan

The most widely used IDO1 inhibitor in biological studies up to date is 1MT [76] despite its moderate activity ($K_i = 19\text{--}53\text{ }\mu\text{M}$) [39, 76–80]. 1MT, like tryptophan, is a chiral compound, and it has been shown that only the L enantiomer (1, Fig. 3)

Fig. 3 IDO1 inhibitors based on the indole scaffold. L-1MT (**1**), D-1MT (**2**), keto-indole (**3**) [81], oxindole (**4**) [66], indole-2-carboxamide (**5**) [67], and 6-fluoro indole (**6**) [53]

inhibits the enzymatic activity of IDO1 in vitro [77, 78]. However, based on the findings that the D enantiomer (**2**, Fig. 3) was more efficacious as an anticancer agent in vivo [78], NewLink Genetics initiated clinical trials with D-1MT (indoximod) as anticancer agent. As a possible explanation of the better in vivo activity of D-1MT it was later suggested that D-1MT preferentially inhibits the IDO1 paralogue IDO2 [27], but this finding was rejected by subsequent studies [82–85]. Therefore, the mechanism of action of D-1MT as a kynurenine pathway inhibitor is still under debate [24, 34, 86–89]. Additional complications in interpreting experimental data generated with the tool compound 1MT may arise from the facts that D-1MT has been shown to induce IDO1 expression [24] and that L-1MT can be substantially contaminated with L-Trp [33].

At present, it is clear that D-1MT is not a human IDO1 inhibitor. The use of L-1MT as a tool compound for IDO1 inhibition should be avoided due to its low activity and specificity, and due to the availability of more active and selective inhibitors.

2.2 Indoles

IDO1 inhibitors known before the implication of this enzyme in tumoral immune resistance were of moderate micromolar activity [90]. Many of these were analogs of the substrate L-Trp or its indole scaffold [76, 77, 91–94]. However, as L-Trp itself shows only a moderate affinity for IDO1 (K_d = 290–320 μ M) [41, 95], also most of its analogs were only moderately active towards IDO1. Double-digit micromolar activities were reached by some keto indoles discovered by virtual screening (**3**, Fig. 3) [81, 96].

Iomet Pharma (now Merck & Co.) tested 3-substituted indoles and oxindoles for IDO1 and TDO inhibition (Fig. 3) [66]. Although none of the compounds displayed an enzymatic IC_{50} value below 100 μ M on IDO1, some were low micromolar IDO1 inhibitors in a cellular context with at least tenfold selectivity over TDO (4), while other compounds were selective for TDO (Sect. 3.1). The observation that compounds display better cellular IC_{50} values than enzymatic IC_{50} values has been frequently observed for IDO1 and could be due to off-target effects or the artificial reducing cofactors routinely used in the enzymatic assay [75].

In the following, Iomet Pharma described a large series of indole-2-carboxamides [67]. Many compounds displayed cellular IC_{50} values below 3 μ M on hIDO1 and more than tenfold selectivity with respect to TDO. For a few compounds, an enzymatic IC_{50} value below 10 μ M was cited (5). Interestingly, the indole-2-carboxamide scaffold has also been shown to possess high antitubercular activity in an animal model of tuberculosis [97, 98], and IDO1 has been implicated in tuberculosis infection [99].

More recently, iTeos Therapeutics described a series of 3-indol-3-yl-pyrrolidine-2,5-diones (succinimides) with potent IDO1 inhibitory properties [53]. The most potent compound (6) in its enantiopure *R* form displayed an enzymatic IC_{50} value of 120 nM, nanomolar cellular activities, an IC_{50} value of 3 μ M in a human whole blood assay, an EC_{50} value of 74 nM in an IDO1-dependent cellular T-cell proliferation assay, in vivo reduction of kynurenine levels in the blood of healthy mice of up to 68% (100 mg/kg), as well as in vivo inhibition of tumor growth in different cancer models.

In summary, it seems to be challenging to develop potent selective IDO1 inhibitors based on the indole scaffold. At the time of writing, only iTeos Therapeutics succeeded in developing a nanomolar indole IDO1 inhibitor.

2.3 Imidazoles

The known heme-binder 4-phenylimidazole (4PI, 7, Fig. 4) was described as an IDO1 inhibitor in 1989 [100] and gained attention due to its co-crystallization with IDO1 in 2006 (Fig. 2a) [41]. A structure-based approach led to 4PI derivatives that were up to tenfold more potent than the parent compound 8 [101]. Fungistatic drugs of the 1-substituted imidazole type, such as miconazole and econazole (9), were discovered in two independent screens to be active against IDO1, while similar 1,2,4-triazole drugs such as fluconazole were completely inactive [102, 103]. Structure-based modifications of the 1-substituted imidazole antifungal scaffold led to more soluble but less potent compounds (10) [103].

Fallarini and coworkers created a virtual library of 1,5- and 4,5-disubstituted imidazoles and selected 25 compounds for synthesis and evaluation [104]. While the 1,5-disubstituted imidazoles were inactive with one exception (11), the best 4,5-disubstituted imidazoles displayed low micromolar enzymatic and cellular IC_{50} values (12). However, the presence of bulky substituents next to the heme-iron

Fig. 4 IDO1 inhibitors with imidazole scaffold. 4-phenylimidazole (4PI, **7**) [100, 101], 4PI derivative (**8**) [101], econazole (**9**) [102, 103], derivative of fungistatic imidazoles (**10**) [103], disubstituted imidazoles (**11**, **12**) [104], 4PI derivative (**13**) [105], and fused imidazole-isoindoles (**14**, **15**, **16**, **17**) [71, 106]. Stereocenters discussed in the main text are marked by an *asterisk*

binding imidazole nitrogen should sterically hinder their direct heme binding, so it is not clear how they bind to IDO1.

NewLink Genetics developed further 4-substituted phenylimidazoles with nanomolar potency but a low ligand efficiency, because they featured a long extension into the B pocket (**13**) [105]. Fusion of the two aromatic rings of 4PI by an aliphatic carbon atom to yield imidazo-isoindoles (**14**), however, strongly enhanced potency with less impact on efficiency [106]. Two compounds of this series (**14**, **15**) were tested in vivo for antitumor activity in mouse models of colorectal, bladder, mammary, and lung cancer as single agents or in combination with other chemotherapies. These tests showed that both compounds had a significant antitumor activity either as single agent or in combination therapy, leading to a significantly reduced rate of tumor growth and improved survival time [106]. Based on these results, it is likely that the clinical candidate GDC-0919 has a structure similar to compounds **14**, **15**. These compounds contain two asymmetric carbon atoms, marked by asterisks in Fig. 4, leading to four stereoisomers. For compound **14**, it was found that

the *S*-isomers of the carbon atom in the tricyclic scaffold were responsible for the nanomolar potency of the mixture of stereoisomers [106].

The pioneering work of NewLink Genetics stimulated other groups to work on the imidazole scaffold. Peng and coworkers evaluated some of the NewLink Genetics compounds and new analogs and provided IDO1-bound X-ray structures for three imidazole-isoindole derivatives and one pyridine analog (Fig. 2d) [43]. The most potent compound described in this work, which corresponds to compound **15** (Fig. 4) [106], was shown to display an enzymatic IC₅₀ value of 19 nM and a cellular IC₅₀ value of 55 nM. Its X-ray structure (PDB ID: 5EK4, Fig. 2d) revealed a hydrogen bond from its hydroxy group to the heme propionate, and the placement of the cyclohexyl group in the B pocket [43]. Here, the A pocket is much higher than in the 4PI-bound structure (Fig. 2a), but this additional volume is apparently neither filled by the ligand nor by solvent molecules. The parent compound without fluoro substituent (**14**), which displayed an enzymatic IC₅₀ value of 38 nM and a cellular IC₅₀ value of 61 nM, was investigated in more detail regarding the activity of its stereoisomers, and it was found that the individual enzymatic IC₅₀ values of the four stereoisomers were 20 nM, 29 nM, 2.4 μM, and >10 μM [43]. Again, the *S*-isomers of the carbon atom in the tricyclic scaffold were found to be responsible for the nanomolar potency of the mixtures of stereoisomers.

Merck Group patented a series of related enantiopure tricyclic imidazo-isoindole compounds of high enzymatic potency (**16**, **17**, Fig. 4, IC₅₀ values < 100 nM) [71], finding the same preference for the *S*-isomers.

In summary, the phenylimidazole scaffold provides a popular and promising starting point for the development of IDO1 inhibitors. Its binding mode to the active site is known through X-ray crystallography [41, 43], it does not show promiscuous enzyme inhibition, rational modifications have been shown to be feasible, and interpretable 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 interactions with IDO1, for example, through molecular recognition by the B pocket. Selective TDO (Sect. 3.2) and dual IDO1/TDO inhibitors (Sect. 4.1) based on this scaffold have also been described.

2.4 *N*-Hydroxyamidines and Structurally Related Compounds

In 2009, Incyte Corporation reported *N*-hydroxyamidines as potent, reversible, competitive IDO1 inhibitors (Fig. 5) [45]. The most potent compound of this series, compound **19**, displayed enzymatic and cellular IC₅₀ values of 67 and 19 nM, suppressed kynurenine generation in vivo, and inhibited melanoma growth in a mouse model [45]. Modeling of the binding of **19** to IDO1 predicted that the oxygen of the hydroxyamidine binds to the heme iron, and that the amidine adopts a *cis* conformation. The phenyl ring was placed inside the A pocket, producing a tight fit, while the

Fig. 5 IDO1 inhibitors based on the N-hydroxyamidine and related scaffolds. Epacadostat (**18**) [107], N-hydroxyamidine (**19**) [45], nitrobenzofurazan N-hydroxyamidine (**20**) [108], N-hydroxyamidines (**21**, **22**) [62], N-phenylamides (**23**, **24**) [65], N-cyclohexylamide (**25**) [65], further modified N-phenylamides (**26**, **27**) [63], benzamide (**28**) [64], phenylurea (**29**) [64], aniline (**30**) [64], and 2-phenyl-propanamide (**31**) [64]

amino substituent on the furazan ring could form a hydrogen bond to the propanoic acid group on the heme ring [45]. The clinical candidate (**18**, epacadostat, CAS# 1204669-58-8) belongs to a further optimized series of N-hydroxyamidines with extension to the B pocket [107, 109]. 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 [38, 46].

Nitrobenzofurazan derivatives of the N-hydroxyamidine scaffold (**20**) [108] showed very good activities in enzymatic and cellular tests but a lower ligand efficiency than the Incyte compound **19**. The described compounds were strongly selective for IDO1 over TDO by factors of 200–1,700.

Flexus Biosciences successfully modified Incyte's N-hydroxyamidine scaffold first by simplifying the presumed B-pocket extension of the N-hydroxyamidines without the furazan ring and preserving nanomolar potencies (**21**, **22**) [62]. Later, they replaced the N-hydroxyamidine group by a simple amide function and to obtain a large number of analogs with cellular IC_{50} values below 50 nM (**23**, **24**, **25**, Fig. 5) [65]. Most modifications again affected the B-pocket extensions, with many potent compounds showing a cyclohexyl-phenyl motif. However, the A pocket also seems to tolerate many changes, for example, the replacement of the phenyl ring by a cyclohexyl ring (**25**).

In two concurrent patent applications, Flexus described further modifications of the scaffold. In one, modifications to the B-pocket extension were mainly based on a cyclohexyl-quinoline motif, potentially with another substituent next to the amide (**26**, **27**) [63]. In the other [64], modifications to the presumed heme-binding moieties are described, such as N-phenylamide to N-benzylamide, N-phenylsulfonamide, benzamide (**28**), phenylurea (**29**), aniline (**30**), and phenylacetamide (**31**, Fig. 5). The nanomolar activities of these compounds with electronically very different heme-facing moieties are quite astonishing and might suggest that the optimal filling of the B pocket plays a crucial role here.

In summary, the N-hydroxyamidine scaffold and related compounds have proven to provide a very promising route to the development of selective IDO1 inhibitors.

2.5 1,2,3-Triazoles

4-Aryl-1,2,3-triazoles were discovered in 2010, with the parent compound (**32**, Fig. 6) displaying an enzymatic IC_{50} value of 60 μ M [44]. The rationally designed triazole **33** (MMG-0358) [110] showed nanomolar activities both in enzymatic and cellular assays, no cellular toxicity, and a high selectivity for IDO1 over TDO [110]. Nanomolar cellular IC_{50} values were also reported for the N-phenyl-1,2,3-triazol-4-amine compounds from Vertex (**34**) [74]. However, bioisosteric replacement of imidazole by 1,2,3-triazole in the NewLink Genetic fused compounds led to only moderately active compounds (**35**) [112].

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. However, no potent compounds with a B-pocket extension have been reported so far.

Fig. 6 Other IDO1 inhibitors with published activity data. 4-phenyl-1,2,3-triazole (32) [44, 110, 111], 1,2,3-triazoles (33, 34) [74, 110], fused 1,2-3-triazole (35) [112], benzenesulfonyl hydrazides (36, 37) [113, 114], triazolothiazole Amg-1 (38) [54], imidazothiazole (39) [42], aminonitrile (40) [59], iminonitrile (41) [61], and 2-amino-phenylureas (42, 43, 44) [55, 56, 115]

2.6 Phenyl Benzenesulfonylhydrazides

A series of phenyl benzenesulfonyl hydrazides (36, Fig. 6) displayed nanomolar IC_{50} values in both enzymatic and cellular assays [113]. In a further development of this scaffold, an optimized lead compound (37) with an enzymatic IC_{50} value of 36 nM, a cellular IC_{50} value of 68 nM, selectivity for IDO1 over 68 other protein targets, good oral bioavailability in rats, significant reduction of the Kyn/Trp ratio in the murine CT26 syngeneic colorectal cancer model, and significant tumor volume reduction in the same model was described [114].

2.7 Fused Thiazoles

The triazolo thiazole (Amg-1, **38**, Fig. 6) was reported by Amgen to inhibit IDO1 with an enzymatic IC_{50} value of 3 μ M, and its selectivity for IDO1 over IDO2 and TDO was demonstrated [54]. Amg-1 was later co-crystallized with IDO1 (Fig. 2b) and used for rational compound optimization. This led to the discovery of imidazothiazole derivatives occupying both pocket A and pocket B [42]. Nanomolar enzymatic inhibition activities were obtained with a urea linker, with the best compound in the series showing an enzymatic IC_{50} value of 77 nM (**39**).

2.8 Aminonitriles and Iminonitriles

Curadev reported aminonitriles as IDO1 inhibitors with enzymatic IC_{50} values below 200 nM [59]. One compound (**40**, Fig. 6) was shown to reduce plasma KYN levels by 40% in mice after inflammatory lipopolysaccharide (LPS) injection used to induce IDO1 expression. Later, the scaffold was modified to feature unsaturated iminonitriles and pyridines (**41**) [61]. For these compounds, only in vivo KYN level reduction values were given, which reached up to 87% after 2 h. Apparently, the predicted promiscuity of this scaffold due to their chemical reactivity as phenolic Mannich bases [75, 116, 117] does not hamper its in vivo use for IDO1 inhibition.

2.9 2-Amino-Phenylureas

In several patent applications, Bristol-Myers Squibb described a 2-amino-phenylurea scaffold and related compounds for IDO1 inhibition yielding low nanomolar to picomolar cellular IC_{50} values (**42**, **43**, **44**, Fig. 6) [55–58, 115]. These are the most potent compounds reported to date, but no data is available about their specificity and mode of action. The three-dimensional structures of the compounds do not seem to be able to fill the IDO1 active site without clashing with surrounding protein residues, suggesting that they could display a novel alternative binding mode.

3 Tryptophan 2,3-Dioxygenase Inhibitors

TDO (EC 1.13.11.11), a functionally related but evolutionarily distant enzyme to IDO1, is constitutively expressed in the liver and regulates systemic L-Trp levels [16]. TDO is a tetrameric enzyme with a higher substrate specificity for L-Trp than IDO1. Recently, it was shown to play a complementary role in different types of cancer [25, 118]. There are nine X-ray structures of TDO available from the RCSB

Fig. 7 X-ray structure of tryptophan 2,3-dioxygenase (TDO) (PDB ID 2NW8) from *xanthomonas campestris* in complex with ferrous heme and tryptophan [120]. Only the active site is shown, displaying the protein in surface representation, heme, selected protein side chains, and resolved solvent molecules in stick representation, and the ligand in ball and stick representation. Heteroatoms are colored as follows: *blue* (nitrogen), *red* (oxygen), and *orange* (iron). Hydrogen bonds are displayed as *thin orange lines*

protein data bank, including a heme-free apo structure of human TDO [119] and a structure from *xanthomonas campestris* in complex with ferrous heme and tryptophan (Fig. 7) [120]. *Xanthomonas campestris* shares a 34% sequence identity with human TDO, and nine out of 11 L-Trp binding residues are conserved [120].

3.1 6-Fluoro Indoles

L-Trp and its analogues 6-F-Trp and 5-F-Trp show a much higher affinity for TDO [120, 121] than IDO1, which facilitated the development of selective TDO inhibitors based on the indole scaffold (Fig. 8).

The oldest and most used TDO inhibitor is the 6-fluoroindole 680C91 (**45**) [122, 123], which displayed a K_i of 30 nM for rat TDO and was shown to be selective for TDO over IDO1 [122, 123]. Its regioisomer 709W92 (**46**) displayed the same K_i for rat TDO, but as it also inhibits serotonin uptake, it has been less used [123, 124]. Recently the K_i of **45** for human TDO was determined to be 880 nM (IC_{50} 280 nM), but it was shown to suffer from limited solubility and bioavailability [25].

Based on these compounds and motivated by the discovery that TDO is implicated in tumoral immune escape, Frédérick and coworkers described a series of more than 70 derivatives of **45**, systematically analyzing and varying all parts of the compound separately [125]. Cellular IC_{50} values on murine TDO were determined and used to develop a structure–activity relationship. Surprisingly, they did not find any compound more potent than **45**, but replacing the pyridine ring by a negatively

Fig. 8 TDO inhibitors based on 6-fluoro indole scaffold. 680C91 (**45**) [122, 123], 709W92 (**46**) [123, 124], LM10 (**47**) [25, 125], pyrazole derivatives (**48**, **49**) [50], indazole derivative (**50**) [52], benzoxazole derivative (**51**) [52], pyridine derivatives (**52**, **53**, **54**) [51], and tetrahydropyridine derivative (**55**) [66]

charged group such as a carboxylate or a tetrazole preserved the favorable interactions of L-Trp with Thr254 (Thr342 in hTDO) and Arg117 (Arg144 in hTDO, Fig. 7) and led to more soluble and stable compounds. In the selected lead compound (**47**), the pyridine ring was replaced by a tetrazole. This compound showed a K_i of 5.6 μ M on a crude extract of human TDO as well as selectivity over hIDO1, type A and B monoamine oxidase, 15 receptors, and three transporters. Unlike the parent compound **45**, it demonstrated a very good solubility and high oral bioavailability in mice [125]. Under the label LM10, **47** was used to show that TDO inhibition could reduce the growth of TDO-expressing P815 mastocytoma cells in mice without producing signs of liver toxicity [25].

Further work of iTeos Therapeutics on the indole scaffold led to the discovery of potent 3-substituted indole compounds with low micromolar or nanomolar enzymatic and cellular activities on hTDO (**48**, **49**, **50**, **51**, **52**, **53**, **54**). In three patents, they describe indoles with different heterocyclic substituents. Besides citing

nanomolar enzymatic and cellular activities, a few compounds were shown to increase circulating L-Trp in mice when administered orally by gavage at 100 mg/kg [50–52]. While pyrazole derivatives were less efficient in this respect (**49**, +40% L-Trp) [50], benzoxazole (**51**, +80% L-Trp) [52] and especially pyridines (**54**, +140% L-Trp) [51] were more efficient.

Iomet Pharma also developed 3-substituted indoles and oxindoles for IDO1 (Sect. 2.2) and TDO inhibition. Three compounds demonstrated nanomolar enzymatic IC_{50} values on TDO and selectivity over IDO1 [66]. The representative compound **55** was selective for TDO, with an enzymatic IC_{50} value of 6.3 μM (IDO1: >130 μM), and a cellular IC_{50} value of 250 nM (IDO1: >100 μM) [66, 126].

Most but not all of these indole-based TDO inhibitors contain a 6-fluoro substituent on the indole ring. For *Xanthomonas campestris* TDO, it has been shown that 6-F-L-Trp has a high affinity for TDO and that it is an efficient TDO substrate [120].

3.2 Imidazoles

While selective IDO1-inhibiting imidazoles are described in Sect. 2.3, and dual IDO1/TDO compounds in Sect. 4.1, TDO-selective imidazole inhibitors have also been discovered (Fig. 9). In the very active imidazo-indole inhibitors developed by Redx Pharma (**56**, **57**, **58**, **59**) [72], the hydroxylated alkyl chain of the imidazo-indole from NewLink Genetics (**62**, Fig. 10) was replaced by an ether or an amino linker. These compounds displayed at least about 20-fold selectivity for TDO over IDO1.

Fig. 9 Other TDO inhibitors. Fused imidazo-indoles (**56**, **57**, **58**, **59**) [72], 4-substituted indazole (**60**) [70], and naphthotriazoledione (**61**) [127]. Stereocenters discussed in the main text are marked by an asterisk

Fig. 10 Dual IDO1/TDO inhibitors. Fused imidazole-indoles (**62**, **63**, **64**) [72, 112], fused imidazole-isindoles (**65**, **66**, **67**) [73], 4-substituted indazoles (**68**, **69**) [70], 3-aminoisoxazolopyridines (**70**, **71**) [128], and furopyridines (**72**, **73**, **74**) [60]. Stereocenters discussed in the main text are marked by an asterisk

3.3 Indazoles

Replacing the indole scaffold by an indazole, Iomet Pharma developed selective TDO and dual IDO1/TDO inhibitors based on the indazol-4-amine scaffold [70]. Many compounds displayed at least 1.5 log units better enzymatic and cellular activities towards TDO than IDO1. Compound **60** is selective for TDO, with an enzymatic IC_{50} value of 85 nM vs. 12 μM .

3.4 Quinones

As early as 1961, it was shown that the quinones or hydroquinones catechol, quinol, p-quinone, L-dihydroxyphenylalanine, and L-epinephrine were able to inhibit TDO [129]. In 2014, a screening of 2,800 compounds from the library of the National Cancer Institute (USA) identified seven compounds inhibiting TDO, among them six quinones such as β -lapachone, taxifolin, nanaomycin A, and mitomycin C [130]. Later, the same group described derivatives of the naturally occurring quinone isatin for TDO and IDO1 inhibition [131]. In these works, only enzymatic assay results were reported, and no additional tests were carried out.

Naphthotriazolediones were discovered as TDO inhibitors based on a structure-based virtual screening [127]. The best compound (**61**, Fig. 9) displayed an IC₅₀ value of 30 nM on hTDO and 20-fold selectivity over IDO. Further investigations of this compound suggested that it does not act through redox cycling and that it is selective for TDO over several other unrelated targets.

4 Dual Indoleamine 2,3-Dioxygenase 1/Tryptophan 2,3-Dioxygenase Inhibitors

As both IDO1 and TDO seem to play complementary roles in cancers, and as their active sites share many similarities, development of dual IDO1/TDO inhibitors is particularly attractive (Fig. 10).

4.1 Imidazoles

After describing fused imidazo-isindole derivatives for IDO1 inhibition (**14**, **15**, Fig. 4), NewLink Genetics also described imidazo-indole derivatives to be dual potent inhibitors of IDO1 and TDO (**62**, **63**, Fig. 10) [112].

Redx Pharma built upon the work of NewLink Genetics, describing nanomolar enzymatic IDO1 and TDO inhibitors based on both the imidazo-indole [72] and the imidazo-isindole [73] scaffolds. They determined the enzymatic IC₅₀ value of one of the four stereoisomers the imidazo-indole compound **62** to be 94 nM [72]. Some new imidazo-indoles, such as, for example, the ether (**64**), seemed to be nearly equipotent towards IDO1 and TDO. The imidazo-isindoles described by Redx (**65**, **66**, **67**) [73] were somewhat more active towards TDO. Incorporation of a pyridine ring into the tricyclic scaffold was found to be beneficial (**65**, **67**), and the placement of a second aromatic ring in the B pocket (**67**) was tolerated.

4.2 Indazoles

The indazol-4-amine scaffold developed by IOMET Pharma yielded selective TDO inhibitors (Sect. 3.3) and dual IDO1/TDI inhibitors [70]. Compounds **68**, **69** displayed enzymatic IC_{50} values below 500 nM towards both IDO1 and TDO.

4.3 3-Aminoisoxazolopyridines

Researchers from the University of Auckland developed very small dual IDO1/TDO inhibitors based on the 3-aminoisoxazolopyridine scaffold (**70**, **71**, Fig. 10) [128]. Compound **70** displayed an enzymatic IC_{50} value of 1–10 μ M and a cellular IC_{50} value below 1 μ M on IDO1, as well as a cellular IC_{50} value of 1–10 μ M on TDO. It reduced the KYN/Trp ratio in plasma and tumors of a glioma mouse model, reduced tumor growth, and led to increased survival when administered in combination with an anti-PD1 antibody [128]. Compound **71** displayed even more potent enzymatic and cellular inhibition of IDO1 and TDO.

Bulky substituents on the pyridine ring as well as substituents of the amino group led to much less active compounds.

4.4 Fuopyridines

Curadev reported di-amino substituted fuopyridines as dual IDO1/TDO inhibitors (**72**, **73**, **74**, Fig. 10) [60]. Here, many compounds displayed enzymatic and cellular IC_{50} values below 200 nM on hIDO1 and enzymatic IC_{50} values below 500 nM on TDO. Two compounds, including compound **72**, also inhibited IDO2 with nanomolar IC_{50} values. Reduction of LPS-induced plasma KYN levels in mice above 50% were reported for a number of compounds. Compounds **72** and **73** were shown to reduce tumor volumes in vivo in the murine CT26 syngeneic colorectal cancer model, to reduce KYN levels in tumor tissue, and to be capable of penetrating the blood–brain barrier [60].

5 Indoleamine 2,3-Dioxygenase 2 Inhibitors

According to recent work [16], human IDO2 is weakly expressed in the liver, testes, and thyroid, but in contrast to previous reports [86, 132] not in human tumors. Two commonly occurring single nucleotide polymorphisms in the coding region of human IDO2 lead to more than 25% of humans having enzymatically inactive IDO2 [27].

Only a few IDO2 inhibitors have been reported (Fig. 11), probably due to the difficulties in expressing and purifying active human IDO2 [133]. L-1MT (**1**, Fig. 3) has been reported to inhibit human IDO2 with K_i values in the range of 300 [85] to 425 μM [134]. In 2010, Incyte reported preliminary results of potent mouse IDO2 inhibitors with more than 100-fold selectivity over mIDO1 but did not disclose their structures [135].

Two years later, a library of 640 FDA-approved drugs were screened in cellular assays on mIDO1 and on mIDO2, and 12 compounds were reported to inhibit mIDO2 at low micromolar concentrations and with selectivity over mIDO1 [102]. For example, the proton pump inhibitor tenatoprazole (**75**) displayed cellular IC_{50} values of 1.8 and $>100 \mu\text{M}$ for mIDO2 and mIDO1, respectively. However, these proton pump inhibitors are known to be prodrugs whose active sulfenamide moiety covalently binds with selected cysteine residues of hydrogen potassium ATPase [136]. Inhibition of IDO1 by cysteine-binding compounds such as ebselen has been demonstrated earlier [137]. Apparent lower inhibition of IDO1 could be due to the fact that the same incubation time was used for both enzymes, but as IDO1 degrades L-Trp much faster than IDO2 [133], this assay was not in its linear phase anymore.

Curadev reported two compounds (**72**, Fig. 10 and **76**, Fig. 11) to inhibit both human IDO1 and human IDO2 in enzymatic assays with IC_{50} values below 1 μM [60].

4-phenyl-1,2,3-triazoles were tested for mIDO2 inhibition in cellular assays, and it was noted that compounds filling only the A pocket were highly selective for IDO1, while compounds filling both A and B pockets displayed similar cellular IC_{50} values for hIDO1 and mIDO2 in the micromolar range (**77**) [133].

Tryptanthrins were also reported as potent hIDO2 inhibitors, but they show much better inhibition of hIDO1 than of hIDO2 (**78**) [134].

In summary, it seems to be challenging to develop selective IDO2 inhibitors, and their practical value has not been unequivocally established.

Fig. 11 Indoleamine 2,3-dioxygenase 2 (IDO2) inhibitors. Tenatoprazole (**75**) [102], furopyridine (**76**) [60], 4-phenyl-1,2,3-triazole (**77**) [133], and tryptanthrine (**78**) [134]

6 Conclusions

Thus far, IDO1 and TDO inhibition has been the main approach to address L-Trp metabolism as therapeutic target. The regulation of their expression might have different effects, as IDO1 has been shown to act also as a signaling protein independently from its enzymatic function [138]. Therapeutic vaccination against IDO1 has demonstrated disease stabilization without toxicity in patients with non-small cell lung cancer (NSCLC) [139]. If tryptophan depletion is the main cause of immune suppression (tryptophan depletion hypothesis), interference with the sensing of low L-Trp levels by the stress kinase GCN2 and the mTOR signaling pathway may provide different access points. Therapeutic regulation of L-Trp levels by modulation of transcellular L-Trp transport might be an alternative [15]. On the other hand, if bioactive L-Trp metabolites such as L-kynurenine (KYN), 3-hydroxy-L-kynurenine (3-HK), kynurenic acid (KA), or quinolinic acid (QUIN) are mainly responsible for immune suppression (tryptophan utilization hypothesis) [8], the inhibition of downstream enzymes along the kynurenine pathway (Fig. 1) such as kynurenine monooxygenase (KMO) or kynurenine aminotransferase II (KAT II) [21] or the suppression of the signaling pathways of the kynurenines, for example, through the aryl hydrocarbon receptor (AhR) [118, 140, 141], could be additional viable targets. Here, knowledge gained from many years of research into neurological disorders may be of value [142].

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