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Review Article

A new cancer immunosuppression target: indoleamine 2,3-dioxygenase (IDO). A review of the IDO mechanism, inhibition and therapeutic applications

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Abstract

Indoleamine 2,3-dioxygenase (IDO) has recently been implicated in tumor immune escape. In particular, IDO undermines a vigorous antitumor immune response by promoting peripheral tolerance, thereby shaping the host environment to be more hospitable to tumor survival and growth. Consequently, the development of potent IDO inhibitors that compromise this toleragenic mechanism is an important therapeutic goal. To assist in the development of more potent IDO inhibitors, the current review presents the proposed catalytic mechanisms of IDO and comprehensively reviews reported IDO inhibitors. Finally, the successful preclinical application of IDO inhibition in a new anticancer modality is described.

Introduction

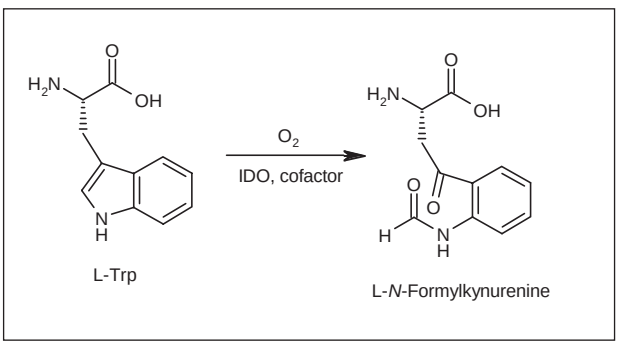
The treatment of advanced (metastatic) cancers is a major clinical challenge. Current regimens involving chemotherapy and other systemic modalities all too often provide only a limited benefit to the approximately 50% of cancer patients in the U.S. and other developed countries who present with advanced disease at diagnosis. Similarly, current regimens ultimately fail patients who relapse with disseminated disease after treatment of their primary tumors. It has long been recognized that tumors display immunogenic tumor antigens yet escape immune destruction, somehow evading or subverting and perhaps even reprogramming the immune system for their own benefit. This phenomenon of "immune escape" is central to tumor cell survival, but its basis is poorly understood (1). An appropriately activated immune system can eradicate cancer, even when it is aggressive and disseminated, but spontaneous occurrences of this are rare. This has prompted the development of numerous peptide- and cell-based anticancer therapies aimed at boosting the immune response (*e.g.*, the administration of cytokines, tumor-associated antigen peptide/vector vaccines, dendritic cell [DC] vaccines and adoptive transfer of tumor antigen-specific effector T-cells expanded *ex vivo* from cancer patients [2-8]). These therapies, which are conceptually based on stimulating components of the immune system that produce an effective response, may not, however, be sufficient to overcome tumor immune escape if pathological immune tolerance is dominant in cancer patients, as has been recently proposed (9).

The enzyme indoleamine 2,3-dioxygenase (IDO, EC 1.13.11.42), which appears to play a key role in protecting allogeneic conceptus from the maternal immune system, has been implicated in the establishment of pathological immune tolerance by tumors. The physiological role of IDO, which catabolizes the essential amino acid tryptophan, has been defined in large part through the

use of the bioavailable IDO-inhibitory compound 1-methyltryptophan (1-MT). This review details current thinking regarding the catalytic mechanism of tryptophan degradation by IDO in conjunction with a comprehensive summary of the current literature on small-molecule IDO inhibitors, and concludes with an overview of how IDO-inhibitory compounds might be incorporated into a novel treatment strategy that has the potential to broadly impact standard cancer therapies.

IDO mechanism

IDO is the first and rate-determining step of the kynurenine pathway of L-tryptophan (L-Trp) metabolism. It catalyzes the addition of oxygen across the C-2/C-3 bond of the indole ring in Trp and generates *N*-formylkynurenine (Scheme 1). *In vitro*, methylene blue and ascorbic acid are a necessary reductant to maintain maximum catalytic activity, but *in vivo* a flavin or tetrahydrobiopterin cofactor is believed to serve this role (10, 11).



Scheme 1: IDO reaction.

The rational design and development of IDO inhibitors requires an understanding of the enzyme's mechanism. Although the exact mechanism of IDO remains unknown, important mechanistic research with IDO and non-enzyme-catalyzed oxidation reactions have led to some understanding of the mechanism and several mechanistic proposals. Much of this work was previously described in an earlier review article (11). The current review will summarize the details in the previous review and provide an update on some recent research.

All the proposed mechanisms begin with the binding of O_2 and Trp at the active site of IDO, although the exact order of binding is uncertain. The active form of IDO has the heme iron in the ferrous (Fe^{2+}) oxidation state and, although the enzyme is prone to auto-oxidation, the primary catalytic cycle does not involve redox changes. The ferric (Fe^{3+}) form of IDO is inactive and requires reduction to the ferrous form before catalytic activity is returned. The ferric form is also particularly susceptible to substrate inhibition by Trp (12).

After O_2 and Trp bind in IDO's active site, all the proposed mechanisms proceed through a 3-indolenylperoxy-Fe^{2+} (**1,** Scheme 2). There are three different proposals for the process to reach **1** (Scheme 2) and there are two different proposed mechanisms for the decomposition of **1** to *N*-formylkynurenine (Scheme 3). Intermediate **1** is central to all the proposed mechanisms because the related 3-hydroperoxyindolenine structure (not shown) has been shown to be a competent intermediate in the nonenzymatic oxidation of Trp to *N*-formylkynurenine (13, 14).

*Three mechanisms for the formation of intermediate **1***

1. Ionic mechanism

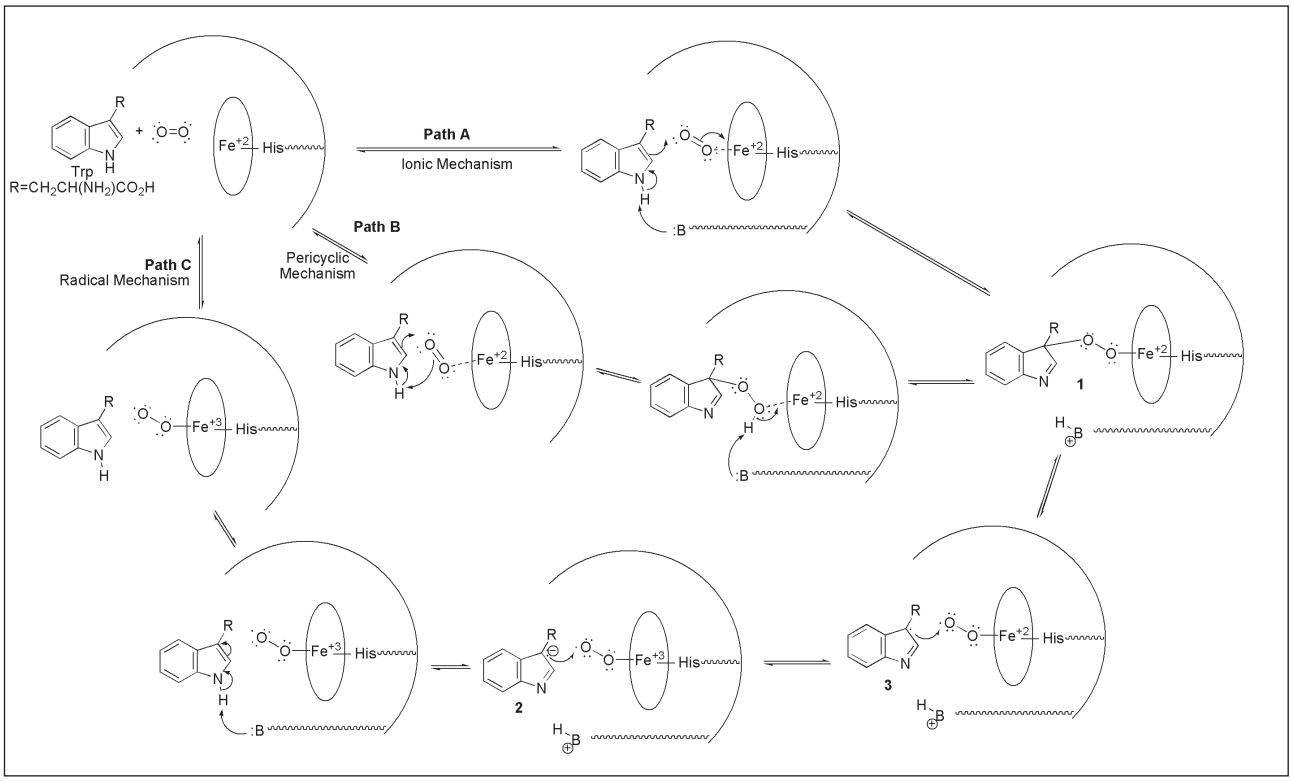
The ionic mechanism (15) has the heme iron serving as a Lewis acid that activates the molecular oxygen (Scheme 2, Path A). Once activated, the electrophilic oxygen undergoes nucleophilic attack by the electron-rich pyrrole portion of the indole to form **1**. Based on substrate and inhibitor studies (16), the N-1 proton of the indole ring of Trp is essential for the oxidation to occur. In the ionic mechanism, it has been proposed that a base in the active site deprotonates the indole to generate a more nucleophilic ring.

2. Pericyclic mechanism

One variant of the ionic mechanism has the distal oxygen of O_2 operating as the basic site (Scheme 2, Path B) (17). Consequently, the reaction is really a pericyclic process and, more precisely, a group transfer reaction similar to an ene reaction (18). Identification of an important basic amino acid in the active site might allow for discrimination between the pericyclic and ionic mechanism. Interestingly, a recent report (19) identified two important amino acids in IDO, His346 and Asp274, through site-directed mutagenesis studies. The authors suggested that the His346 might be the proximal heme iron ligand. The role of the Asp274 is unknown, but the authors suggest it might serve as the distal heme ligand or be important for conformational stability. It is also possible that one of these amino acids is the general base for the deprotonation of the N-1 indole proton.

3. Radical mechanism

The third mechanism (20) (Scheme 2, Path C) involves a one-electron process to reach intermediate **1** and, similar to the ionic mechanism, involves deprotonation of the N-1 indole proton by a base in the active site. However, in the radical mechanism, the indole anion **2** undergoes a one-electron oxidation to generate the intermediate **3**. The two radical structures at the active site can combine to generate **1**.



Scheme 2: Three proposed mechanisms to 3-indolenylperoxy-Fe^{2+} (**1**).

*Two mechanisms for the transformation of **1** to N-formylkynurenine*

1. Criegee-type rearrangement

After formation of the key 3-indolenylperoxy-Fe^{2+} intermediate **1**, two possible pathways are proposed. The first and more likely is a concerted Criegee-type rearrangement to afford the labile cyclic hemiacetal intermediate **4** (Scheme 3, Path D) (15, 21). Simple decomposition of **4** leads to formylkynurenine.

2. Dioxetane retrocycloaddition

Alternatively, **1** may have the distal oxygen add to the C-2 position of the indole to form the dioxetane intermediate **5** (Scheme 3, Path E) (15, 21). A retrocycloaddition of **5** yields *N*-formylkynurenine. Since the formation of the strained intermediate **5** would be thermodynamically unfavorable, this pathway is considered less likely. Furthermore, the highly exothermic decomposition of **5** should lead to light emission, but chemiluminescence has never been detected in the enzyme reaction. Nonenzymatic mechanistic studies also undermine the dioxetane intermediate pathway (22, 23).

The addition of other nucleophiles to the C-2 position of the indole in **1** has also been proposed. Notably, the α-amino group of Trp might add to generate a tricyclic intermediate, 3a-hydroperoxypyrrolo[2,3-*b*]indole derivative (**6**, Fig. 1), which subsequently undergoes conversion to *N*-formylkynurenine with expulsion of the α-amino group. Evidence for the existence of **6** has been found in the nonenzymatic oxidation of Trp (13), but not in the process catalyzed by IDO. Amino acid side-chain residues at the active site have also been proposed to transiently add to the C-2 position of the indole, although no experimental evidence exists to support this idea (23).

IDO inhibitors: chemistry and pharmacology

Structural classes of IDO-inhibitory molecules

There exists only a small collection of reports describing inhibition studies of IDO. Not surprisingly, the studies have focused primarily on derivatives of Trp and structurally related heterocycles like β-carboline, despite

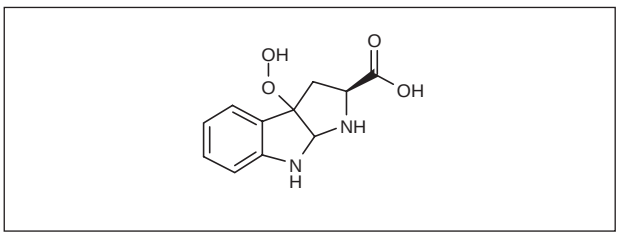
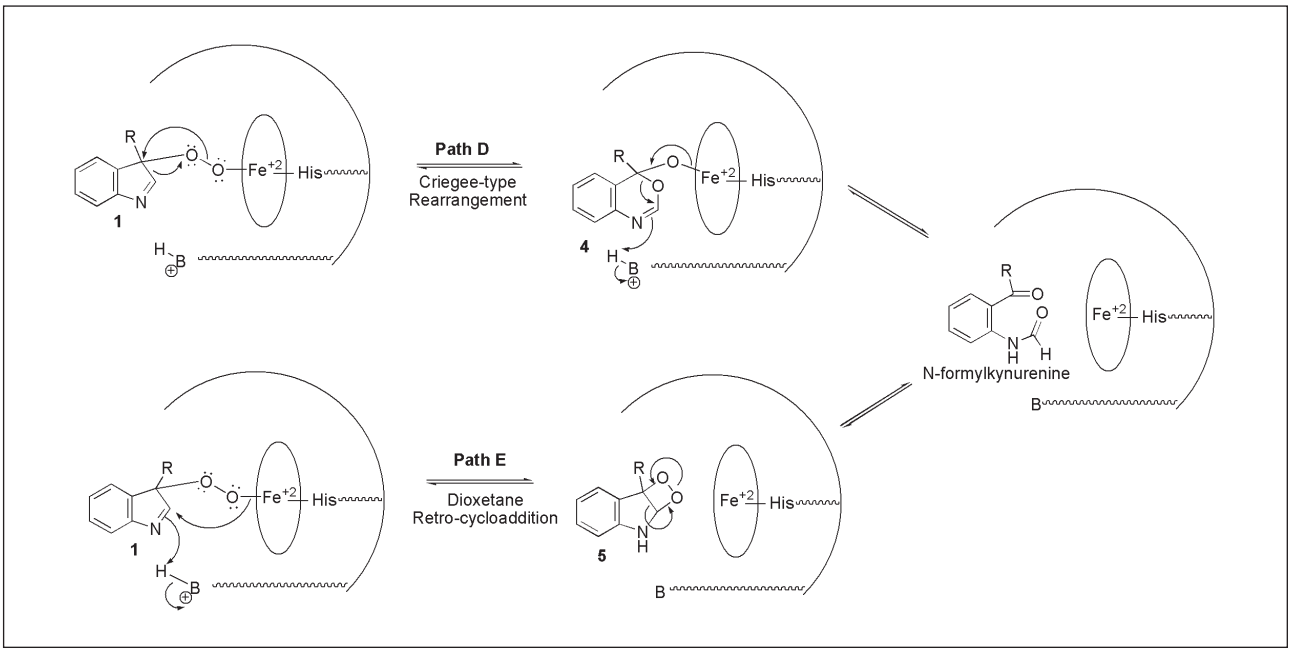


Fig. 1. 3a-Hydroperoxypyrrolo[2,3-*b*]indole derivative (**6**).



Scheme 3: Two proposed mechanisms to *N*-formylkynurenine.

the reported (24-26) promiscuity of IDO compared to the related tryptophan 2,3-dioxygenase (TDO2, EC 1.13. 11.11). Both competitive and noncompetitive inhibitors of IDO have been identified. To date, competitive inhibitors are primarily derivatives of Trp, while noncompetitive inhibitors are derivatives of β-carboline; both contain an indole core.

Competitive inhibitors

Substrate inhibition with high concentrations (> 0.2 mM) of L-Trp was reported (12, 27) during early enzymological studies, and therefore it is not surprising that Trp derivatives have been extensively studied. Derivatization of the Trp structure has occurred in three areas: substitution of the indole ring, modification of the amino acid sidechain and modifications of the indole ring.

1. Tryptophan indole ring substitutions

Substitution of the indole ring of Trp has afforded the most commonly used inhibitor of IDO: 1-MT (**7,** Table I) (16). A racemic mixture was originally used by Munn and coworkers in their seminal study of the fetal survival paradox (28), but subsequent studies (29) with isolated IDO have revealed a slight preference for the natural (*S*)- (L) isomer of **7** (the more precise Cahn-Ingold-Prelog system of configurational assignment will subsequently be used in preference to the historic D,L system). Furthermore, the (*S*)-isomer of the natural substrate Trp has 10-50 times smaller K_m values than (*R*)-Trp (30). Stereochemical preference for the natural isomer was also reported with the 6-nitro derivative **24** (Table I). On the other hand, some cellular studies (31-33) demonstrate greater activity for the (*R*)-(D) isomer of **7** (1-MT). Given the more complex nature of cellular studies, IDO- inhibitory activity may not be the primary reason for the better activity of the (*R*)-isomer of **7**. Nevertheless, based on these conflicting results future inhibition studies should carefully consider both stereoisomers of Trp analogues.

Table I comprehensively summarizes the range of substituents that have been tested on the indole ring of Trp. The seven most potent compounds based on the reported inhibition data are the five monosubstituted derivatives, 1-methyl (**7**), 5-bromo (**15**), 6-fluoro (**23**), 6-nitro (**24**), ([*S*]-isomer), 7-fluoro (**26**), and the two difluorinated derivatives 4,7-difluoro (**14**) and 5,7-difluoro (**21**). Excluding the 1-methyl derivative, the other six are electron-withdrawing groups (34-36). Since the proposed mechanisms for IDO-catalyzed conversion of Trp to *N*-formylkynurenine (see above) all begin with electron donation from the pyrrole ring of Trp, electron-withdrawing groups on the indole ring would make this step less favorable and slower. Nevertheless, the activity data in Table I indicates that the 5-bromo (**15**) and the 6-fluoro (**23**) derivatives undergo oxidation; therefore some of these compounds still behave as substrates despite their deactivating substitution.

Several compounds, notably the 5-bromo (**15**) and 2-hydroxy (**12**) derivatives, have significantly different IDO inhibition values reported by different sources. Some of the variability may be due to the different IDO sources and assay conditions used in the different studies. Peterson and coworkers extracted IDO from human monocyte/macrophage cells induced by interferon gamma (29). They monitored IDO activity by detecting kynure-

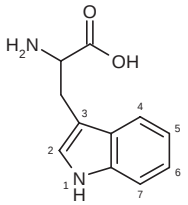


Table I: Trp derivatives with indole ring substitution.

Compound	Indole ring substition	Stereochemistry at α position	Inhibition data (%)[a]	Activity data (%)[b]	Ref.
7	1-CH_3	*S* (L)	52.3 (62.9)[c]; K_i = 34 μM[c]		29
7	1-CH_3	*R,S*	26; K_i = 6.6 μM[d]	7	37
7	1-CH_3	*R* (D)	5.7 (11.6)[c]		29
8	1-CH_2CH_3	*S*	13.5 (9.9)[c]		29
9	1-SO_2Ph, 6-OCH_3	*R*	3.2 (28.4)[c]		29
10	2-Cl	*S*	20	33	37
11	2-Br	*S*	11	21	37
12	2-OH	*S*	30	4	37
12	2-OH	*R,S*	-38.4 (-43.3[c]		29
13	4-CH_3	*R,S*	26	33	37
14	4-F, 7-F	*S*	K_i = 40 μM		11
15	5-Br	*R,S*	0[c]		29
15	5-Br	*R,S*	56	36	37
16	5-CH_3	*R,S*	6	123	37
17	5-OCH_3	*R,S*	35	70	37
18	5-OCH_2Ph	*R,S*	2	1	37
19	5-OH	*S*	12	59	37
19	5-OH	*S*	14[c]		29
20	5-F	*R,S*	32	46	37
21	5-F, 7-F	*S*	K_i = 24 μM		11
22	6-CH_3	*R,S*	20	72	37
23	6-F	*R,S*	54	38	37
24	6-NO_2	*S*	52	2	37
24	6-NO_2	*R*	7	0	37
25	7-CH_3	*R,S*	36	18	37
26	7-F	*S*	K_i = 37 μM		11

[a] Unless otherwise stated, inhibition data are reported as 100 minus percent of tryptophan metabolized in an *in vitro* competitive inhibition assay with 1 mM of inhibitor. Figures in parentheses indicate inhibition data with 2-h preincubation of inhibitor with IDO.
[b] Percent compound oxidized relative to L-tryptophan.
[c] 100 μM inhibitor concentration used in inhibition assay.
[d] K_i determined at pH 8.0 in reference (16).

nine product with a radioimmunoassay or HPLC assay. Southan and coworkers used recombinant human IDO, expressed in and purified from *Escherichia coli* (37). They followed IDO activity with a spectrophotometric assay that detected an imine derivative of kynurenine. Several inhibitors reported in subsequent tables were evaluated against IDO isolated from rabbit small intestine using two different detection methods (16, 38). Despite these differences, several compounds show striking consistency, *i.e.,* **7** and **19** (Table I) and **45** (Table II).

Several electron-releasing substituents in Table I are very active as substrates and are oxidized by IDO: 4-methyl (**13**), 5-methyl (**16**), 5-methoxy (**17**), 5-hydroxy (**19)** and 6-methyl (**22**). One derivative (5-methyl, **16**) is more active than L-Trp. This result is consistent with the mechanistic rationale and the outcome described for the electron-withdrawing substituents. Electron-releasing substituents would be expected to make the indole ring more nucleophilic, leading to a faster initial reaction with the oxygen species at the active site.

The 1-methyl derivative **7** defies the trend seen with substituents on the benzene portion of the indole ring. The proposed mechanisms (see above) for IDO involving pyrrole electron donation actually initiate the reaction with deprotonation of the N-1 hydrogen of Trp. Without a hydrogen, **7** prevents the deprotonation from occurring. Similar inhibition is seen with benzofuran (**54**) and benzothiophene (**55**) analogues of Trp (Table III; see below). However, there is a limited amount of space in the active site to accommodate N-1 groups, as the 1-ethyl (**8**) and 1-phenylsulfonyl (**9**) derivatives exhibited only weak inhibitory activity.

Indole ring substitution of Trp derivatives has been extensively explored; nevertheless, the use of multiple

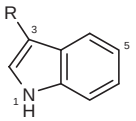


Table II: Trp side-chain modifications.

Compound	R[a]	Stereochemistry at α position	Inhibition data (%)[b]	Activity data (%)[c]	Ref.
27	-$CH_2CH_2NH_2$		28	32	37
28	-$CH_2CH_2NH_2$; {5-OCH_3}		-43.9[d]		29
29	-$CH_2CH_2NH_2$; {2-CO_2H}		16.3 (17.9)[d]		29
30	-$CH_2CH_2NH_2$; {2-CO_2H , 5-OCH_3}		10.8 (3.4)[d]		29
31	-$CH_2CH_2CO_2H$		0	8	37
32	-$CH_2C(CH_3)(NH_2)CO_2H$	*R,S*	1	35	37
33	-$CH_2CH(NHCH_3)CO_2H$	*S*	33	21	37
34	-$CH_2CH(NHCOCH_3)CO_2H$	*S*	7	3	37
35	-$CH_2CH(NH2)CO_2CH_3$	*S*	30	15	37
36	-$CH_2CH(NH_2)CO_2CH_2CH_3$	*S*	7	14	37
37	-$CH_2CH(OH)CO_2H$	*R,S*	9.7 (1.4)[d]		29
38	-$CH_2N(CH_3)_2$		-6.6[d]		29
39	-CH_2CN		3.5 [d]		29
40	-$CONH_2$; {5-OH}		0[d]		29
41	-CHO		4.4[d]		29
42	-$CH=CHCO_2H$		2.5 (3.2)[d]		29
43	-$CH=CHCO_2CH(CH_3)_2$		15.2 (11.6)[d]		29
44	-(*E*)-CH=CH-(3-pyridinyl); {6-F}			0	40
45	-$CH(CH_3)CH(NH_2)CO_2H$	α-*S*,β-*S*;α-*R*,β-*R*	0.0 (-2.7)[d]		29
45	-$CH(CH_3)CH(NH_2)CO_2H$	α-*S*,β-*R*;α-*R*,β-*S*	9.8 (3.6)[d]		29
45	-$CH(CH_3)CH(NH_2)CO_2H$	*R,S*	7	32	37
46	-CH_2-5'-(3'-methyl-2'-thioxo-4'-imidazolinone)	*R,S*	K_i = 11.4 μM		39
47	-$CH_2CH(NH_2)CO$-(*S*)-Trp	*S*	K_i = 147 μM		29

[a] Additional indole substituents are added in brackets.

[b] Unless otherwise stated, inhibition data are reported as 100 minus percent of tryptophan metabolized in an *in vitro* competitive inhibition assay with 1 mM of inhibitor. Figures in parentheses indicate inhibition data with 2-h preincubation of inhibitor with IDO.

[c] Percent compound oxidized relative to L-tryptophan.

[d] 100 μM inhibitor concentration used in inhibition assay.

substituents is a strategy that might yield more active inhibitors. Excluding compounds **9**, **14** and **21**, few compounds with multiple substituents have been synthesized and tested. The synthetic challenge posed by polysubstituted indoles is probably one reason that these examples are limited. Another limitation would appear to be the space available in the indole binding region of the active site, as seen in the weak activity and inhibition with **18**. Despite these limitations, it is clear that a range of substituents has been accommodated and therefore combinations of these might afford synergistic inhibition. Unlike the β-carboline derivatives (see below), there has been no indication of slow-binding inhibition from Trp derivatives; the preincubation inhibitory data in Tables I-III do not substantially differ from the percent inhibition found in standard competition assays.

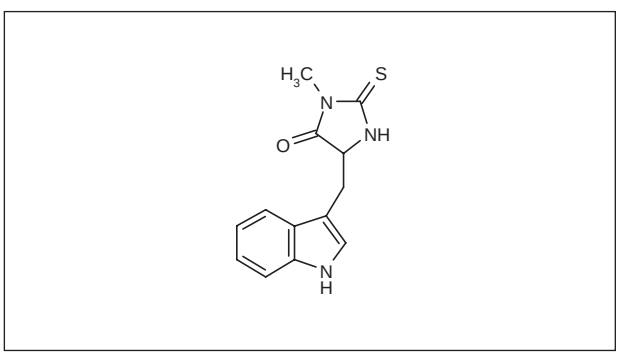


Fig. 2. Compound **46**.

2. Tryptophan side-chain modifications

A range of Trp side-chain modifications have been explored, as illustrated in Table II. However, relatively few of these have afforded compounds with promising inhibition. Modest inhibition was realized with the addition of a methyl group to either the α-amine (**33**) or the α-acid (**35**). One notable derivative with interesting activity and a novel structure is the thiohydantoin derivative (**46**) (39). Further modification of the thiohydantoin ring might provide even more potent inhibitors.

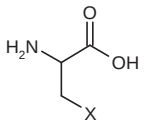


Table III: Indole ring modifications of Trp.

Compound	X	Stereochemistry at α position	Inhibition data (%)[a]	Ref.
48	3-(1*H*-indazolyl)-	R,S	0.0	29
49	3-(7-azaindolyl)-	R,S	-1.6	29
50	3-indolinyl	S	0.4 (3.0)	29
50	3-indolinyl	R	-2.4 (-1.2)	29
51	3-quinolinyl	S	0	29
51	3-quinolinyl	R	0	29
52	(2-aminophenyl)methyl	S	-0.3	29
53	(2-amino-3-hydroxyphenyl)methyl	R,S	-0.4	29
54	3-benzofuranyl	R,S	43[b,c]	37
54	3-benzofuranyl	R,S	K_i = 25 μM	16
55	3-benzothiophenyl	R,S	16[b,d]	37
55	3-benzothiophenyl	R,S	K_i = 70 μM	16
56	1-(1,4-cyclohexadienyl)	S	K_i = 230 μM	41

[a] Unless otherwise stated, inhibition data are reported as 100 minus percent of tryptophan metabolized in an *in vitro* competitive inhibition assay with 100 μM of inhibitor. Figures in parentheses indicate inhibition data with 2-h preincubation of inhibitor with IDO.

[b] 1 mM inhibitor concentration used in inhibition assay.

[c] 22% of **54** was oxidized by IDO.

[d] 19% of **55** was oxidized by IDO.

3. Indole ring modifications

Modifications of the indole ring have afforded a few novel competitive inhibitors (Table III). Most notable among this group are the benzofuran (**54**) and benzothiophene (**55**) derivatives described earlier. These two compounds, like 1-MT (**7**), lack an N-1 proton and therefore cannot be deprotonated, the initial step in the proposed catalytic mechanism of IDO indole oxidation (11, 17). Attempts at identifying feedback inhibition from subsequent intermediates in the kynurenine pathway failed with the kynurenine analogue (**52**) and the 3-hydroxykynurenine analogue (**53**). Surprisingly, based on the success of electron-withdrawing groups on the benzene portion of the indole (Table I), a pi-deficient analogue of indole, 7-azaindole (**49**), also failed to demonstrate inhibitory activity. Similarly, modifications of either the pyrrole portion of the indole ring, *i.e.*, reduction (**50**) or incorporation of another nitrogen (**48**), also failed to afford inhibition. The majority of the data from Table III indicate that the indole ring is almost essential for the creation of a competitive inhibitor.

4. Miscellaneous structures

A small selection (Table IV) of structures unrelated to Trp have been tested for competitive inhibition. Similar to the modified indole ring structures in Table III, the majority of the structures have not shown any inhibitory activity. Feedback inhibition was not detected with kynurenic acid (**60**) or quinolinic acid (**63**), nor was inhibition seen with the structurally related analogues **59**, **61** and **62**. Two interesting exceptions were discovered with **58** and **64**. 3-Amino-2-naphthoic acid (**58**) is an analogue of anthranilic acid, an intermediate in the aromatic pathway of Trp metabolism. Although assay differences preclude direct comparisons of the potency of IDO inhibitors, compound **58** is one of the most potent inhibitors yet reported in the literature. It is clearly one of the most interesting lead compounds, notwithstanding the synthetic challenge of constructing 3-amino-2-naphthoic acid analogues. A second unique inhibitor was pyrrolidine dithiocarbamate (**64**) (42). This antioxidant demonstrated notable inhibitory activity against IDO generated from interferon gamma treatment of human monocyte-derived macrophages. It is possible that the sulfur of the dithiocarbamate is binding to the heme iron in the active site of IDO. This binding mode would be consistent with sulfur's well-known affinity for iron in biological systems, *e.g.*, ferrodoxin.

Noncompetitive inhibitors

The first class of structures exhibiting IDO inhibition was a series of β-carboline structures reported in 1984 (38). Initially, they were reported to exhibit uncompetitive inhibition, but β-carboline (**65**), also known as norharman,

Table IV: Other compounds tested for competitive inhibition.

Compound	Structure	Inhibition data (%)[a]	Ref.
57	1-amino-2-naphthoic acid	-2.0 (11.2)	29
58	3-amino-2-naphthoic acid	74.2 (75.2)	29
59	3-quinolinecarboxylic acid	-2.6	29
60	4-hydroxy-2-quinolinecarboxylic acid	1.1	29
61	4,8-dihydroxy-2-quinolinecarboxylic acid	2.9	29
62	2-picolinic acid	1.5	29
63	quinolinic acid	6.8	29
64	pyrrolidine dithiocarbamate	44[b]; IC_{50} = 6.5-12.5 μM	42

[a] Unless otherwise stated, inhibition data are reported as 100 minus percent of tryptophan metabolized in an *in vitro* competitive inhibition assay with 100 μM of inhibitor. Figures in parentheses indicate inhibition data with 2-h preincubation of inhibitor with IDO.
[b] 125 mM inhibitor concentration used in inhibition assay.

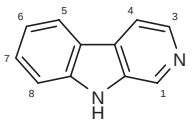


Table V: β-Carboline ring substitution compounds.

Compound	β-Carboline ring substitution	Inhibition data (%)[a]	Ref.
65	none	50.3 (57.0); K_i = 178 μM	44
66	3-OCH_2CH_3	5.5 (21.2)	44
67	3-$OCH_2CH_2CH_3$	16.7 (76.7) ; K_i = 98.0 μM	44
68	3-OCH_2CH_2OH	6.7 (11.0)	44
69	3-CO_2t-Bu	7.0 (7.2); K_i = 89.7 μM	44
70	3-$COCH_2CH_2CH_3$	-4.1 (44.9)	44
71	3-NH_2	0.9 (-19.4)	44
72	3-N=C=S	26.7 (86.1)	44
73	3-OH	30.1 (-5.3)	44
74	3-CO_2CH_3, 6-F	40.4 (49.2); K_i = 7.4 μM	44
75	3-CO_2CH_3, 6-Br	-4.9 (13.4)	44
76	3-CO_2H	K_i = 40.6 μM	44
77	3-CO_2CH_3	K_i = 259 μM	44
78	3-$CO_2CH_2CH_2CH_3$	K_i = 98.0 μM	44
79	3-$CH_2CH_2CH_2CH_3$	K_i = 3.3 μM	44
80	3-NO_2	K_i = 37.5 μM	44
81	3-$CO_2CH_2CH_3$, 6-F	K_i = 21.0 μM	44
82	3-CO_2CH_3, 6-N=C=S	K_i = 8.5 μM	44
83	1-CH_3, 7-OCH_3	10[b]	38
84	1-CH_3, 2-O, 7-OCH_3	46[c]	38
85	1-CH_3, 7-OH	-11[b]	38
86	1-CH_3	-13[b]	38
87	1-CO_2CH_3, 7- OCH_3	25[b]	38
88	1-CH_3, 7-OCH_3, 3,4-dihydro	4[b]	38
89	1-CH_3, 7-OH, 3,4-dihydro	21[b]	38
90	1,2,3,4-tetrahydro	0[c]	38
91	1-OH, 7-OCH_3, 3,4-dihydro	-13[c]	38

[a] Unless otherwise stated, inhibition data are reported as 100 minus percent of tryptophan metabolized in an *in vitro* competitive inhibition assay with 100 μM of inhibitor. Figures in parentheses indicate inhibition data with 2-h preincubation of inhibitor with IDO.
[b] 2 mM inhibitor concentration used in inhibition assay with rabbit intestine IDO.
[c] 1 mM inhibitor concentration used in inhibition assay with rabbit intestine IDO.

was subsequently reclassified as a noncompetitive inhibitor (43). β-Carboline derivatives (Table V) continue to be the most common type of noncompetitive inhibitor, but three novel structures (Table VI) have also been reported (44).

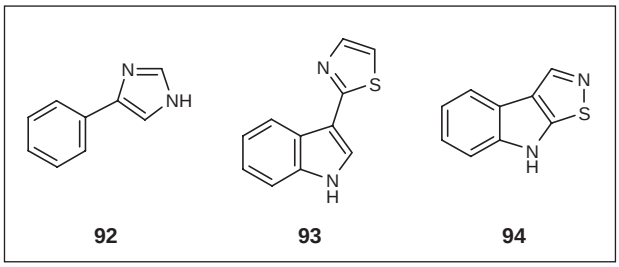
1. β-Carboline derivatives

Modifications to the β-carboline structure have occurred in both the pyridine and the benzene rings. The pyridine ring has been reduced and substituted at C-1 and C-

Table VI: Other compounds demonstrating noncompetitive inhibition.

Compound	Structure	Inhibition data (%)[a]	Ref.
92	4-phenylimidazole	K_i = 4.4 μM	43
93	camalexin	21.3	44
94	brassilexin	K_i = 5.4 μM	44

[a] Unless otherwise stated, inhibition data are reported as 100 minus percent of tryptophan metabolized in an *in vitro* competitive inhibition assay with 100 μM of inhibitor.



92 **93** **94**

3, and the benzene ring has been substituted at C-6 and C-7. There are still many positions of the β-carboline structure that have not been explored. The most potent IDO inhibitors have larger alkyl substituents in the C-3 position, *e.g.,* **79** and **81**. There appears to be a hydrophobic pocket in the active site capable of accommodating these alkyl groups. Fluorine and the isothiocyanate group were present in several potent C-6-substituted β-carboline derivatives, *e.g*., **74**, **81** and **82**.

As noncompetitive inhibitors, β-carboline derivatives do not compete for the same active site location as Trp or other indoleamine substrates. Nevertheless, there is experimental evidence that indicates that β-carboline (**65**) binds directly to the heme iron at the active site as a nitrogen ligand and competes with oxygen for binding at the active-site iron (43). Sono has determined that the β-carboline occupies another binding site close to the L-Trp binding region and he hypothesizes that this space may be available for a natural cofactor or a regulator of the enzyme (30). Interestingly, several of the β-carboline inhibitors, such as **67**, **70** and **72**, demonstrated considerably greater potency on preincubation with IDO. This is suggestive of slow binding inhibition and may indicate these inhibitors need time to settle into the second binding pocket near the heme iron. One important liability of β-carboline derivatives is the reported neuroactivity of these structures as benzodiazepine receptor ligands (45-48). In fact, many previous IDO inhibitor studies were focused on developing treatments for neurological disorders (*e.g.*, excitotoxic brain lesions) where penetration of the central nervous system may have been necessary. However, an IDO inhibitor able to penetrate the central nervous system could cause problematic side effects in cancer therapy.

2. Miscellaneous structures

A small group of other compounds have been discovered to be noncompetitive inhibitors. Although limited in number, these structures provide some unique and potent structural leads. 4-Phenylimidazole **92** is believed to bind to the heme iron, similar to β-carboline (**65**) (30). It seems possible that brassilexin (**94**) may also bind to the heme iron through the sulfur of the isothiazole ring.

Although a selection of compounds have been investigated for IDO inhibition, submicromolar inhibition has not yet been achieved. A few unique structures have been discovered to have IDO-inhibitory activity, athough the majority of the most active structures contain the indole core or resemble L-Trp. Clearly, one important goal in the development of IDO inhibition as a cancer therapy will be to discover more potent inhibitors, and it seems that a diversification of IDO inhibitor structures may be necessary to achieve this goal.

Therapeutic potential of IDO inhibitors

IDO suppresses activation of T-cell immunity

IDO is an extrahepatic enzyme that catalyzes the initial and rate-limiting step in the degradation of tryptophan along the kynurenine pathway that leads to the biosynthesis of NAD^+ (nicotinamide adenine dinucleotide) (26, 49). IDO does not, however, handle dietary catabolism of tryptophan, which is instead the role of the structurally unrelated liver enzyme tryptophan dioxygenase (TDO2). Moreover, NAD^+ levels in mammalian cells are predominantly maintained by salvage pathways. Thus, for many years the biological role of IDO remained unclear. Recently, however, it has been demonstrated that IDO modulates immune function by suppressing cytotoxic T-cell activation (reviewed in 50). The physiological relevance of IDO-mediated immunosuppression was confirmed in a seminal study which demonstrated that administration of the bioactive IDO inhibitor 1-MT (16) can elicit MHC-restricted, T-cell-mediated rejection of allogeneic mouse concepti (28, 51).

Genetic control of IDO by the tumor suppressor gene Bin1

Elevated tryptophan catabolism in cancer patients, first reported in the 1950s (52), was generally ascribed to be a tumoricidal effect of interferon gamma elevation operating through IDO to starve the rapidly growing tumor cells of the essential amino acid tryptophan (53). However, the elucidation of IDO's toleragenic role has recently prompted the opposing hypothesis that elevated IDO can promote tumor cell immune escape by suppressing the activation of cytotoxic T-cells that could recognize and destroy them.

A key finding in support of this hypothesis has been the discovery of a regulatory link between IDO elevation

in tumor cells and an established cancer suppression signaling pathway controlled by the adaptor protein Bin1 (39). Bin1 was originally identified through its ability to interact with and inhibit the oncogenic activity of the c-Myc oncoprotein (54, 55). Subsequent studies have indicated complex splice regulation of Bin1 protein isoforms in cells, which are linked to diverse cellular processes, and systemic disruption in homozygous *Bin1* knockout mice results in perinatal lethality associated with severe cardiomyopathy (56). Existing studies in human prostate and breast cancers support the candidacy of *Bin1* as a tumor suppressor or negative modifier gene. Loss or attenuation of *Bin1* expression occurs in > 50% of primary human breast tumors and in all breast tumor cell lines examined to date (57). The 2q14-21 region, where *Bin1* is located, is frequently deleted in breast cancers (58, 59), particularly in tumors that contain *BRCA1* mutations or have metastatic capacity (58-61). In prostate cancers, *Bin1* shows frequent loss of heterozygosity (LOH) and loss of expression, especially in advanced cases with metastatic capacity (62, 63). Studies utilizing *Bin1* knockout mouse-derived cell lines corroborate the hypothesis that *Bin1* has an antiprogression role in cancer. In particular, *Bin1* loss provides a dramatic cell-extrinsic benefit to *in vivo* tumor growth that is explained by IDO dysregulation (39).

Preclinical studies combining IDO inhibitors with breast cancer chemotherapy

Based on these pivotal studies linking *Bin1* loss to IDO upregulation and immune escape by tumors, critical proof-of-principle studies have been performed. These studies have led to the discovery of a novel therapeutic strategy whereby IDO inhibitors in combination with standard chemotherapeutic agents cooperatively produce dramatic regression of established tumors in preclinical studies (39). One prediction of the hypothesis framed in the previous section is that an IDO inhibitor might break immune tolerance and promote tumor regression. We employed the well-accepted mouse model of breast cancer, the MMTV-*Neu* "oncomouse" that develops mammary gland tumors closely resembling human ductal carcinoma *in situ* (DCIS), to investigate this idea. The possible antitumor effects of the well-established IDO inhibitor 1-MT were evaluated either alone or in combination with other agents. 1-MT treatment alone slowed tumor growth but did not reverse it, consistent with other recently published observations (64, 65). This finding suggests that single-agent IDO inhibitor-based immunotherapy has limited antitumor efficacy when applied to established tumors. In contrast, treatment of tumor-bearing MMTV-*Neu* mice with a combination of 1-MT + paclitaxel produced an average decrease of approximately 30% in tumor volume at the 2-week endpoint, while paclitaxel treatment by itself produced only tumor growth inhibition. Histological and immunohistochemical examination revealed evidence of increased cell death in tumors from mice treated with 1-MT + paclitaxel (39). Consistent with host immunity being critical for the therapeutic regression of tumors, immunodepletion of either $CD4^+$ T-cells (39) or $CD8^+$ T-cells (unpublished) abrogated the ability of 1-MT to cooperate with paclitaxel. Similar cooperativity was observed with some but not all chemotherapeutic agents tested (39).

In summary, IDO inhibition produces dramatic antitumor efficacy in the autochthonous MMTV-*Neu* tumor model when combined with certain cytotoxic chemotherapeutic agents. This finding is striking as it supports what has generally been viewed as a counterintuitive notion, that combining immunotherapy with chemotherapy can be used to effectively promote tumor regression. Immunotherapy and chemotherapy have generally been thought to work at cross purposes; however, the case for complementarity has been gaining credence as of late, based on experiments employing increasingly sophisticated models and tools to monitor the progress of antitumor immune responses (66).

While a useful tool for proof-of-principle studies, 1-MT is not the ideal compound for development as a clinical agent. 1-MT is a weak inhibitor, especially in cell-based assays (where the EC_{50} is > 200 μM), and has poor solubility characteristics. To address these issues, we have conducted enzyme- and cell-based screens of commercially available compounds and have identified a series of thiohydantoin derivatives of tryptophan that are pharmacologically superior to 1-MT. The most potent of these is the compound 3'-methylthiohydantointryptophan (termed MTH-trp), with an EC_{50} in the cell-based assay of 12 μM (39), being approximately 20-fold more potent than 1-MT.

A trial of MTH-trp in the MMTV-*Neu* autochthonous tumor model revealed that it has biological activity similar to or better than that of 1-MT. Over the 2-week course of treatment, MTH-trp alone promoted tumor growth delay but in combination with paclitaxel promoted tumor regression. MTH-trp combination therapy achieved an overall reduction in mean tumor volume over the 2-week treatment period of 58% (including one complete regression) as compared to 30% with 1-MT. As with 1-MT combination therapy, tumor regression was found to be associated with increased tumor cell death (39).

Therapeutic potential of IDO inhibitors based on market analysis

Cancer is the second leading cause of death in the U.S., with over 500,000 people dying each year. Of the approximately 1.4 million new cases of cancer diagnosed in 2004, 563,700 will have died in 2004 (41% death rate). Of these deaths, 28% will have been from lung cancer, 10% from colon cancer, 7% from breast cancer and 5% from prostate cancer. These cancers, often referred to as "The Big Four", represent the major targets to positively impact the cancer survival rate. Breast cancer is the most frequently diagnosed cancer in women. In 2002, approximately 200,000 new cases were diagnosed in the U.S.

Table VII: U.S. market for cancer therapies.

Therapy	1995	2000	2005*
Cytotoxic drugs	$1,397	$1,863	$1,975
Antimetabolites	$265	$338	$355
Antitubulin agents	$580	$783	$846
Alkylating agents	$220	$281	$295
Others	$305	$378	$412

and over 1 million new cases worldwide. Treatment for breast cancer is correlated to the disease stage and patient hormone receptor status, with specific therapy decisions based on an individual patient's likelihood of response. Typical interventions for breast cancer range from surgical resection, for the treatment of localized stage 1 tumors, to combination treatment strategies that employ surgery, radiotherapy and multidrug therapy for patients with advanced breast cancer. Current treatment of metastatic (stage IV) disease, in particular, must balance prolonging the patient's life with the impact of treatment on the patient's quality of life. In breast cancer, fewer than 15% of patients who develop metastatic disease survive for 5 years after diagnosis, irrespective of treatment.

The extraordinary size of the cancer market becomes apparent in Table VII, which shows sales (in millions) of chemical anticancer drugs in the U.S., with 2005 projected (as denoted by the asterisk).

The market for cancer chemotherapies in 2001 reached $4 billion in the U.S. and $10.8 billion worldwide, and is growing at an annual rate of 10%. Of this market, breast cancer therapies represented approximately $250 million annual sales in the U.S. The antitubulin segment, of which Taxol® (paclitaxel) is a member, is projected to be the fastest growing segment of the cancer therapies market. Taxol® sales reached $1.2 billion in 1998 and $1.7 billion in 2001. The expanded use of Taxol® (and the newer taxanes), as represented by the over 20% per year increase in sales since its introduction, reflects both its utility and the very limited alternatives available for the effective treatment of solid tumors. IDO inhibitors, which are likely to work most effectively as immunomodulatory adjuncts to conventional chemotherapeutics, represent particularly attractive candidates for clinical development in this context. As such, IDO inhibitors have tremendous potential to cooperatively leverage taxane-based breast cancer therapy, as well as other chemical therapies for a variety of cancer indications.

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