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Synthesis and evaluation of oxindoles as promising inhibitors of the immunosuppressive enzyme indoleamine 2,3-dioxygenase 1†

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Indoleamine 2,3-dioxygenase 1 (IDO1) is considered as an important therapeutic target for the treatment of cancer, chronic infections and other diseases that are associated with immune suppression. Recent developments in understanding the catalytic mechanism of the IDO1 enzyme revealed that conversion of L-tryptophan (L-Trp) to N-formylkynurenine proceeded through an epoxide intermediate state. Accordingly, we synthesized a series of 3-substituted oxindoles from L-Trp, tryptamine and isatin. Compounds with C3-substituted oxindole moieties showed moderate inhibitory activity against the purified human IDO1 enzyme. Their optimization led to the identification of potent compounds, **6**, **22**, **23** and **25** (IC₅₀ = 0.19 to 0.62 μM), which are competitive inhibitors of IDO1 with respect to L-Trp. These potent compounds also showed IDO1 inhibition potencies in the low-micromolar range (IC₅₀ = 0.33–0.49 μM) in MDA-MB-231 cells. The cytotoxicity of these potent compounds was trivial in different model cancer (MDA-MB-231, A549 and HeLa) cells and macrophage (J774A.1) cells. Stronger selectivity for the IDO1 enzyme (124 to 210-fold) over the tryptophan 2,3-dioxygenase (TDO) enzyme was also observed for these compounds. These results suggest that the oxindole moiety of the compounds could mimic the epoxide intermediate state of L-Trp. Therefore, the structural simplicity and low-micromolar inhibition potencies of these 3-substituted oxindoles make them quite attractive for further investigation of IDO1 function and immunotherapeutic applications.

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Introduction

Indoleamine 2,3-dioxygenase 1 (IDO1) and tryptophan 2,3-dioxygenase (TDO) enzymes are the two important heme-containing redox enzymes that catalyse the initial and rate-determining step for the transformation of L-Trp to N-formylkynurenine through the kynurenine pathway.^{1–3} IDO1 is expressed in several tissues all throughout the body, but over-expressed in a variety of non-hepatic tissues, including the lungs, epididymis, gut and placenta. Cytokines like interferon-γ are mostly accountable for this over-expression of IDO1 enzymes in non-hepatic tissues.¹ TDO is expressed predominantly in hepatic tissues and its activity regulates L-Trp balance in response to dietary intake. The increased level of IDO1 expression is interrelated with reduced prognosis in different cancers, including pancreatic, ovarian and others.^{3,4}

However, it was recently reported that endothelial IDO1 expression in kidney tumours is associated with better prognosis.^{5,6} Stimulated expression of IDO1 is also related to neurodegenerative disorders, age related cataracts, HIV, encephalitis and others.^{4,7,8} Depletion of the local concentration of L-Trp and generation of kynurenine and other metabolites including, the excitotoxin quinolinic acid, N-methyl-D-aspartate receptor antagonist kynurenic acid, and nicotinamide adenine dinucleotide (NAD) help IDO1 restrain local immune response by perturbing the proliferation of T-lymphocytes.^{4,9}

Cancer immunotherapy by targeting the IDO1 enzyme is recognized as an exciting approach for drug development. Unlike TDO the active site of the IDO1 enzyme is amenable to small molecules. Recent studies in different animal models of cancer described that the perturbation of IDO1 activity by using small molecules successfully restrained the abnormal growth of tumours. This IDO1 inhibition approach also showed augmented effects with chemotherapeutic and radio-therapeutic treatment of malignant tumours.^{10,11} Currently, IDO1 inhibitors INCB024360 and NLG919 in combination with humanized antibodies are under clinical trials for the treatment of cancer and other diseases.^{12,13} The tryptophan-based compound, D-1-MT, is also under clinical

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trial as a kynurenine pathway inhibitor, but its mechanism of action is uncertain. It is described that high concentrations of D-1-MT restrain kynurenine generation, but it fails to successfully reinstate IDO1-promoted obstruction of T-cell proliferation and other biological activities.^{4,14} IDO1 is also a promising therapeutic target for the treatment of chronic viral infections and others that are related to pathological immune suppression.^{13,15} Therefore, there is a clear and unmet need to develop small molecule-based IDO1 inhibitors that will adequately address this cancer immunotherapeutic approach.

There are several known L-Trp or indole-based IDO1 inhibitors. 1-Methyl-L-tryptophan (L-1-MT) is one of the commonly used IDO1 inhibitors but with moderate activity. Brassinin, tryptamine, carboline, keto-indoles, indol-2-yl ethanones, 1-methyl-tryptophan-tirapazamine, isatin and other indole derivatives also showed poor to moderate IDO1 inhibitory activities.^{16–22} Comprehensive mechanistic studies of IDO1 induced L-Trp catabolism revealed that the addition of ferrous heme-iron coordinated molecular oxygen to the C2–C3 double bond of the pyrrole ring is the prerequisite for the IDO1 supported oxidation of L-Trp. These studies also proposed the formation of an epoxide intermediate state during the transformation of L-Trp to *N*-formylkynurenine by the IDO1 enzyme.^{23–25} Therefore, development of Trp or indole-based IDO1 inhibitors that could block this enzyme-dependent oxidative cleavage reaction of the pyrrole ring could construct mechanism-based effective IDO1 inhibitors (Fig. 1).

In an attempt to find effective IDO1 inhibitors, we synthesized oxindole derivatives and explored their enzyme inhibition potential. Several of our tested oxindole-based compounds showed low-micromolar inhibitory activities against the purified IDO1 enzyme. Selected compounds also showed low-micromolar IDO1 enzyme inhibitory activity in MDA-MB-231 cells and almost no/negligible cytotoxicity. Additional studies showed that these potent compounds were more se-

lective toward the IDO1 enzyme in comparison with the TDO enzyme, making oxindoles of compelling value for further development as therapeutic agents targeting IDO1.

Result and discussion

Synthesis of oxindole derivatives

Earlier, L-Trp derivative 5-((1*H*-indol-3-yl)methyl)-3-methyl-2-thioxoimidazolidin-4-one, 1-methyltryptophan-tirapazamine and tryptamine derivatives were reported as moderate inhibitors of the IDO1 enzyme.⁴ However, no L-Trp analog showed low-micromolar activities, probably because of the moderate affinity of L-Trp for the IDO1 enzyme ($K_d \sim 300 \mu\text{M}$).⁴ We hypothesize that L-Trp derivatives that could mimic the transition/intermediate state of the enzymatic reaction offer a better approach in developing potent IDO1 inhibitors. In this regard, we developed oxindole derivatives of L-Trp and tryptamine. Recently, several oxindole derivatives have been reported as potent inhibitors of TAK1 kinase and activators of AMP kinase.^{26,27} Oxindole derivatives also show anti-glycation, antifungal, and other biological activities.^{28,29} Oxindole-based alkaloids from *Uncaria tomentosa* have been described to suppress L-Trp degradation.^{30,31} Therefore, the oxindole moiety could be considered as a useful tool in drug discovery.

A thorough understanding of the IDO1 assisted transformation of L-Trp to *N*-formyl-kynurenine recommends that the presence of a carbonyl group at the C2-position of the pyrrole ring of L-Trp could perturb further radical addition of the Fe(III)-superoxide intermediate (Fig. 1).²³ Consequently, oxindole derivatives could hinder the IDO1 enzyme-dependent oxidative cleavage reaction of the pyrrole ring of L-Trp. Therefore, we hypothesized that oxindole-based derivatives could be used as potent inhibitors of the IDO1 enzyme (Fig. 1).

Initially, we synthesized oxindole derivatives 1 and 2 of L-Trp and tryptamine, respectively by treating with an oxidizing mixture of DMSO/HCl in AcOH solution.³² To explore the role of hydrophobic substitution of the amine groups, oxindole derivatives of L-Trp and tryptamine 3, 4, 5, 6, and 7 were synthesized from *N*-Cbz, *N*-Boc, *N*-benzoyl and *N*-acetyl protected L-Trp and tryptamine, respectively, under similar experimental conditions. To understand the importance of free acid groups, compounds 8 and 9 were synthesized from only *C*-methyl and both *C*-benzyl and *N*-CbZ protected L-Trp (Scheme 1).³² For the same purpose, 2-amino-3-(2-oxoindolin-3-yl)propanamide (10) was also synthesized from compound 3 according to the reported procedures.^{33,34} To explore the role of C3-substitution of the oxindole moiety in inhibiting IDO1 enzyme activity, we also synthesized the hydrazone (compound 11–16), phenylimino (17–19) and alkene-oxindole (20 and 21) derivatives of isatin. The C3-substituted 3-hydroxy-3-alkyl derivatives of isatin and 5-chloroisatin were synthesized according to the reported procedure to explore the role of substitution in the oxindole ring in IDO1 enzyme activity.³⁵

Fig. 1 Proposed epoxide intermediate for the IDO1 induced metabolism of L-Trp (A and B) and its mimic (C).

Table 1 Inhibitory activity of the L-Trp and tryptamine-based 2-indolinone derivatives against the purified human IDO1 enzyme

Compound	IDO1 inhibition IC ₅₀ ^a (μM)	Compound	IDO1 inhibition IC ₅₀ ^a (μM)
3.39 ± 0.29		2.31 ± 0.21
3.97 ± 0.39		13.59 ± 0.19
1.28 ± 0.28		2.25 ± 0.23
1.95 ± 0.32		8.33 ± 0.21
1.58 ± 0.27		385.41 ± 35.31
0.62 ± 0.11		

^a IC₅₀ values are the mean of five independent assays.

appears that the presence of a 4-chloro substituent (12) is favourable for the inhibitory activity with IC₅₀ values of 0.63

μM (Table 2). Meanwhile, the 2-Br substituted phenylimino derivative of isatin 19 showed stronger IDO1 inhibitory

Table 2 Inhibitory activity of the isatin-based oxindole derivatives against the purified human IDO1 enzyme

Compound	IDO1 inhibition IC ₅₀ ^a (μM)	Compound	IDO1 inhibition IC ₅₀ ^a (μM)
1.95 ± 0.29		0.93 ± 0.06
0.63 ± 0.15		1.69 ± 0.13
1.51 ± 0.15		0.36 ± 0.08
3.74 ± 0.32		0.19 ± 0.07
1.58 ± 0.12		4.73 ± 0.61
1.78 ± 0.39		0.45 ± 0.09
2.85 ± 0.42		1.97 ± 0.41
2.56 ± 0.22		0.89 ± 0.07
1.24 ± 0.38		

^a IC₅₀ values are the mean of five independent assays.

activity ($IC_{50} = 1.24 \mu M$) among the tested phenylimino compounds (Table 2). These results suggest that halogen substitution on the aryl ring of the hydrazone and phenylimino derivatives of the oxindoles also plays an important role in their IDO1 inhibition activity. The effect of halogen substitution on the oxindole ring was also explored for probable interactions with the hydrophobic residues present within pocket-'A' (comprises Y126, F163 and S167 residues) of the IDO1 enzyme. Several research groups had successfully taken advantage of these hydrophobic interactions in the optimization of the IDO1 inhibition efficacies.^{4,12,13} The alkene oxindole derivatives of isatin and 5-chloroisatin showed moderate to strong inhibitory activity (IC_{50} values of 0.19 to 1.69 μM). Compound 23 showed a considerably higher IDO1 enzyme inhibition potency ($IC_{50} = 0.19 \mu M$) among the tested compounds. We tested the 3-hydroxy-3-alkyl derivatives of isatin and 5-chloroisatin for further improvement of the efficacy of the oxindoles. Our activity assay showed that the 3-hydroxy-3-alkyl-oxindole derivatives of isatin and 5-chloroisatin had moderate IDO1 inhibition activities (IC_{50} values 0.45 to 4.73 μM) among the tested oxindoles. Therefore, both 5-chloro and suitable substitutions at the C3-position of the oxindole ring had considerable effects on IDO1 enzyme inhibition activity. However, the 3-hydroxy-3-alkyl derivatives do not exhibit substantial effects on the IDO1 inhibition efficacy. Recently, the 3-hydroxy-3-alkyl isatin derivatives were developed as comparable inhibitors of both IDO1 and TDO enzymes.²¹ This also endorses the importance of C3-substituted oxindole moieties in designing inhibitors for these enzymes.

The inhibition efficacies of these oxindole derivatives were determined by a spectrophotometric method. Therefore, additional IDO1 activity assays for the selected compounds were performed by HPLC analysis according to the reported procedures.¹² The level of L-Trp catabolism by the IDO1 enzyme in the absence or presence of the potent inhibitors was monitored by HPLC analyses after quenching the reaction by acidification and hydrolysis of *N*-formylkynurenine to kynurenine (Table S1 in the ESI†). A standard curve was generated using pure kynurenine and then the amount of formed kynurenine from L-Trp in the absence or presence of the compounds under enzymatic reaction conditions was measured to investigate their inhibitory efficacies. The calculated concentrations of the tested compounds required to inhibit kynurenine generation from L-Trp under similar experimental conditions were in the low-micromolar range and in accordance with the inhibition, the activities were calculated using a *p*DMAB-method.¹² The differences in IC_{50} values of the compounds between the spectroscopic and HPLC-based methods could be due to the methylene blue-ascorbate regeneration system, which preserves IDO1 in its active state (Fe^{2+}). The IC_{50} values from the HPLC-based assay also revealed that the presence of a carbonyl group at the C2-position of the 5-membered pyrrole ring is beneficial for the inhibitory activity of the tested compounds, whereas an appropriate substituent at the C3-position of the oxindole ring assists the compounds in strongly interacting with the IDO1 enzyme.

Spectroscopic studies for binding analysis of 2-indolinones with IDO1. The UV-Vis absorption properties of the porphyrin-ring are highly sensitive and useful in understanding the ligand/substrate's binding ability to the IDO1 enzyme.^{41–44} The UV-Vis absorption spectra of ferric-IDO1 and deoxy-ferrous-IDO1 were recorded in the absence and presence of compounds 3, 6, 23, and 25 (Fig. 2A and B). The absorption spectrum of only ferric-IDO1 showed a Soret peak at 404 nm, which is in accordance with the reported results.^{13,42–45} In the presence of compounds, this Soret peak showed a slight blue shift with an increase in intensity, under similar experimental conditions. Compound 25 showed a maximum blue shift of 9 nm, indicating its strong binding to the ferric-IDO1 enzyme, possibly through the carbonyl oxygen of the oxindole-ring (Fig. 2A). The blue-shift of the Soret peak could be associated with the presence of an electron-withdrawing group close to the porphyrin-ring, which is also in accordance with their mode of interaction. Fig. 2B showed that in the absence of any inhibitor the deoxy-ferrous-IDO1 enzyme exhibits Soret and Q-bands at 421 and 558 nm.^{13,42,43} However, in the presence of inhibitors the Soret band shifted to 419–425 nm and new Q bands around 527/558 nm appeared (Fig. 2B). This indicates their probable binding to the Fe^{2+} -IDO1 enzyme possibly *via* the carbonyl oxygen of the oxindole-ring. The absorption spectra of only the compounds did not show any peak in this region (spectra not shown here). Although further studies are required to prove the coordination of the oxindole derivatives to the heme-group of IDO1, the results strongly support the proposed mimic of the epoxide intermediate state for the L-Trp oxidation by these oxindoles.

Cellular IDO1 inhibitory activities of oxindoles. To explore the therapeutic potential of these oxindoles, six of the most potent compounds were tested for their IDO1 cellular activity in MDA-MB-231 breast cancer cells. It is well documented that interferon (IFN)- γ appreciably induces the expression of the native human IDO1 enzyme from its mRNA in MDA-MB-231 cells.^{12,46} The cellular inhibition activities of the compounds are in accordance with those of *in vitro* data against the purified IDO1 enzyme. The calculated EC_{50} values of the

Fig. 2 Absorption spectra of ferric-IDO1 (A) and deoxy-ferrous-IDO1 (B) enzymes in the absence and presence of 50 μM compounds in 100 mM potassium phosphate buffer at pH 6.5. The Y-axis is not the same for both spectra. IDO1 enzyme concentration = 5 μM . The ferrous-deoxy reaction environment was generated by adding $Na_2S_2O_4$ under a N_2 atmosphere.

compounds are within a 0.33–1.26 μM range in MDA-MB-231 cells (Table 3). The control compound, L-1-MT, showed EC_{50} values of 120 μM under similar experimental conditions.⁴⁷ The differences in the EC_{50} values of the compounds between the enzymatic assay against purified IDO1 and cellular assays could be due to the difficulty of controlling IDO1 redox activity, which maintains IDO1 in its active state (Fe^{2+}) and/or environmental effects. Overall, a good correlation between these assays corroborates the IDO1 inhibition potencies of these oxindoles. MTT assay of the compounds in MDA-MB-231 (breast cancer), A549 (lung cancer), HeLa (cervical cancer) and J774A.1 (macrophage) cells (concentrations of IC_{50} and $2 \times \text{IC}_{50}$ values from the enzymatic assay) also revealed no/negligible level of toxicity of the compounds under the tested conditions (Fig. S1–S5 in the ESI†).

Mode of IDO1 enzyme inhibition by the potent oxindoles.

To understand the mode of IDO1 inhibition by the compounds, we determined the enzyme kinetics in the presence of eight potent compounds 3, 6, 12, 19, 22, 23, 25, and 27. The plots of $1/V$ vs. $1/[S]$ showed that compound 12 and 27 followed uncompetitive inhibition and 3, 6, 19, 22, 23 and 25 followed competitive inhibition modes (Fig. 3 and S6†). $[S]$ and V represent the substrate concentration and initial rate of reaction, respectively. Detailed mechanistic studies for the IDO1 mediated transformation of L-Trp to N-formylkynurenine showed that formation of the ferric superoxide intermediate due to the binding of O_2 to the $\text{Fe}(\text{III})$ of the heme-group is the prerequisite for this oxidation reaction. Therefore, although these tested compounds follow a competitive/uncompetitive mode of inhibition with respect to L-Trp, they may not be competitive/uncompetitive with respect to O_2 . For this reason, additional kinetics measurement studies with respect to O_2 are required to understand the comprehensive mode of IDO1 inhibition by these compounds.

Probable mode of interaction of the potent oxindoles with IDO1 enzyme. With confirmation that potent oxindoles inhibit IDO1 enzyme activity, we performed molecular docking analyses of the three potent compounds to explore their probable mode of interaction with the IDO1 enzyme (PDB code: 2D0T).⁴⁸ Molecular docking analysis revealed that the potent compounds 6, 23 and 25 presumably interact with the

Fig. 3 Determination of the mode of inhibition of the potent compounds. Plot of $1/V$ against $1/[S]$ for compounds 3 (A), 6 (B), 22 (C) and 23 (D). Concentration of L-Trp was varied from 50 to 150 μM . The concentrations of the compounds were varied from 200 to 1500 nM. All absorption measurements were performed in 50 mM phosphate buffer pH 6.5 at room temperature.

IDO1 enzyme in a similar pattern to L-Trp (Fig. 4).⁴⁷ The interaction energy of compounds 6, 23 and 25 is $-153.18 \text{ kJ mol}^{-1}$, $-142.33 \text{ kJ mol}^{-1}$, $-146.22 \text{ kJ mol}^{-1}$ respectively. The oxindole-moiety of the compounds is presumably placed in pocket-'A' and interacts with the Ser167 residue through H-bonding. Hydrophobic amino acids like Phe163, Phe164, Tyr126 and others present in pocket-'A' could be involved in pi-stacking or halogen bonding and hydrophobic interactions with the oxindole moiety. Recently, it was reported that hydrophobic residues Phe163 and Phe164 are important for the activity of the IDO1 enzyme.⁴⁹ Meanwhile, polar residues Ser167 and Tyr126 are crucial for the high L-Trp affinity of the IDO1 enzyme.⁵⁰ Therefore, the modes of interaction clearly suggest that the oxindole-moiety is mimicking the intermediate state for the L-Trp oxidation. The model structures also predict that the carbonyl group of the

Table 3 IDO1 enzyme inhibitory activity of the selected compounds in MDA-MB-231 cells

Compound	IDO1 inhibition in MDA-MB-231 cell ^a EC_{50} ^b (μM)
3	1.26 ± 0.17
6	0.48 ± 0.12
12	0.42 ± 0.14
22	0.46 ± 0.11
23	0.33 ± 0.09
25	0.49 ± 0.19
L-1-MT ^c	119.66 ± 11.31

^a IDO1 protein expression in MDA-MB-231 cells was induced by human IFN- γ (20 ng mL^{-1}). ^b EC_{50} values are the mean of three independent assays. ^c Reported compound.

Fig. 4 Probable mode of interaction of the compounds 6 (A), 23 (B) and 25 (C) with the active site of the IDO1 enzyme (2D0T). The model structures were generated using MoleGro Virtual Docker, version 6.0. The oxygen and nitrogen atoms are shown in red and blue, respectively. Residues involved in interactions through hydrogen bond formation are shown using dashed lines (yellow). Images were generated using PyMol.

oxindole ring is orientated towards the heme-group, which is in accordance with our predicted mode of interaction of these mechanism-based inhibitors. The substituents at the C3-position may interact with IDO1 through different modes. The free α -carboxyl groups of compound 6 could be involved in H-bonding with Ser263 and Ala264 residues, whereas its acyl-protected α -amino group could be involved in interactions with the heme-group. The carbonyl (from acetylacetone) of the alkene oxindole derivative 23 could be involved in H-bonding with the Ser263 and Ala264 residues. 5-Chloro substitution in the oxindole ring could assist compound 23 in interacting with the residues in pocket-'A' through halogen bonding, pi-stacking and hydrophobic interactions. For compound 25 the carbonyl (from acetylacetone) and 3-hydroxyl groups could be involved in the interactions with Ser263 and Ala264 residues. Recently, the 3-hydroxy-3-alkyl derivatives of isatin were developed as favourable inhibitors of the TDO enzyme. This study also showed that the 3-hydroxy group plays a crucial role in the proper binding of the inhibitors within the active site of the TDO enzyme.²¹ Therefore, H-bonding, hydrophobic and other interactions play important roles in the stronger binding of the oxindole derivatives of L-Trp to the IDO1 active site. Molecular docking interaction energies of the oxindoles 6, 23 and 25 are not in complete agreement with the measured IC_{50} values of these compounds.

The differences in the IC_{50} values among compounds 6, 23 and 25 could be due to their efficiency in properly fitting into the binding pocket. During docking analysis the binding pocket was considered as a 'rigid-body'. In solution the IDO1 binding pocket may have different conformations, which may possibly explain their different IC_{50} values. Therefore, the mode of interaction of these compounds with IDO1 could be different or the volume/interaction pattern of the C3-substituents could be crucial for their binding under the experimental conditions. Overall, molecular docking analyses support that suitable C3-substituted oxindoles may act as a mimic of the epoxide intermediate for the transformation of L-Trp to N-formylkynurenine by the IDO1 enzyme.

Inhibitory activities of oxindoles against purified TDO enzyme. TDO is the other enzyme, which also catalyses the initial, rate-limiting step of the kynurenine pathway. TDO also catabolizes more than 90% of the L-Trp in the liver to regulate its level. To investigate the efficacy of these oxindoles in selectively inhibiting the IDO1 enzyme, TDO enzyme inhibition studies were also performed (Table 4). Screening against the purified TDO enzyme revealed that these oxindoles were considerably inactive ($IC_{50} \geq 20 \mu M$) against the TDO enzyme. Substitution at the 3-position of the oxindole ring also plays a significant role in inhibiting TDO enzyme activity. Selected oxindole derivatives of L-Trp and tryptamine showed 18 to 135-fold stronger IDO1 inhibition in comparison with the TDO enzyme under similar experimental conditions. The oxindole derivatives of isatin showed 20 to 200-fold stronger IDO1 inhibition in comparison with the TDO enzyme. The most effective oxindole-based compound, 23 (based on the enzymatic and cellular assays), exhibited over a 200-fold increase in inhibitory activity toward the IDO1 enzyme when compared against the TDO enzyme. Recently, a series of 3-hydroxy substituted isatin derivatives were developed as inhibitors for the TDO enzyme. However, our studies suggest that suitable C3-substituents are crucial in the proper binding with IDO1 over the TDO enzyme.²¹

In this study, oxindole derivatives were designed as mechanism-based inhibitors of the IDO1 enzyme. Subsequent modification at the C3-position of the oxindole ring led to the identification of potent inhibitors with low-micromolar IDO1 enzyme inhibitory activities under *in vitro* conditions. The low-micromolar IC_{50} values of the compounds and smaller differences in their inhibitory activities suggest that the presence of an oxindole-moiety could be the driving force for their moderate potencies. Overall, activity studies showed that N-acyl protected oxindolealanine (6) could considerably enhance the inhibition potency of the L-Trp derivatives.

Suitable substitution and restricted rotation at the C3-position of isatin/5-chloroisatin could also augment their inhibition potencies. The UV-Vis absorption properties of the

Table 4 Inhibitory activity of the selected compounds against purified human IDO1 and TDO enzymes

Compound	Mode of IDO1 inhibition	IDO1 inhibition ^a (IC_{50} (μM))	TDO inhibition ^a (IC_{50} (μM))	Selectivity ratio ^b
1	NM	3.39 ± 0.29	60.22 ± 2.02	18
3	Competitive	1.28 ± 0.28	27.20 ± 3.08	21
6	Competitive	0.62 ± 0.11	83.47 ± 3.27	135
7	NM	2.31 ± 0.21	58.33 ± 3.58	25
12	Uncompetitive	0.63 ± 0.15	53.38 ± 0.51	85
13	NM	1.51 ± 0.15	46.81 ± 4.21	31
15	NM	1.58 ± 0.12	38.31 ± 4.56	24
16	NM	1.78 ± 0.39	52.90 ± 3.31	30
19	Competitive	1.24 ± 0.38	46.24 ± 6.06	37
20	NM	0.93 ± 0.06	71.01 ± 5.09	76
22	Competitive	0.36 ± 0.08	54.69 ± 2.59	152
23	Competitive	0.19 ± 0.07	39.92 ± 1.03	210
25	Competitive	0.45 ± 0.09	56.12 ± 6.14	124

^a IC_{50} values are the mean of three independent assays against purified enzymes. ^b Selectivity ratio is calculated as (IC_{50} value of TDO)/(IC_{50} value of IDO1). NM = not measured.

heme-containing porphyrin-ring in the absence and presence of the compounds suggest the interaction of the carbonyl oxygen of the oxindole-ring with the heme-group of the IDO1 enzyme. The molecular model structures also suggest that additional hydrogen bond interactions of the oxindole ring with the Ser167 residue along with the interaction with the residues present in "pocket-A" of the IDO1 enzyme could be the primary contributing factor for the stronger inhibitory properties. The potent compounds also exhibited a 15 to 210-fold stronger inhibition in the IDO1 enzyme compared to the TDO enzyme. Until now limited indole/tryptophan-based derivatives have been reported with such selective and stronger IDO1 enzyme inhibition properties.^{16–22} These results clearly suggest that designing compounds, which could perturb the electrophilic addition of O₂ of the oxygenated-heme group to the pyrrole ring of L-Trp, could be an efficient approach to improve the potency as well as the selectivity of IDO1 inhibitors.

Conclusion

In summary, we synthesized oxindole derivatives with moderate inhibitory activities against the purified human IDO1 enzyme. Activity measurements showed that the oxindole ring plays an important role in their IDO1 inhibitory potencies. Halogen substitution and restricted rotation around the C3-position of the oxindole ring were effective in improving their efficacies. Spectroscopic studies supported the interaction of the potent compounds with the heme-group of the IDO1 enzyme, indicating its preliminary role in mimicking the epoxide-intermediate for the IDO1 catalysed transformation of L-Trp to *N*-formylkynurenine. IDO1 activity in the interferon- γ -induced MDA-MB-231 cells showed that the tested compounds have minimal cytotoxicity and low-micromolar potencies. These oxindole derivatives also showed selectivity for the IDO1 enzyme over the TDO enzyme. Overall, these observations suggest that the oxindole derivatives are potential inhibitors of the IDO1 enzyme, which could be of interest as a drug target in cancer and other human diseases.

Experimental

General information

All reagents were purchased from different commercial sources and used directly without further purification. Column chromatography was performed using 60–120 mesh silica gel. Reactions were monitored by thin-layer chromatography (TLC) on silica gel 60 F254 (0.25 mm). ¹H NMR and ¹³C NMR spectra were recorded at 400 and 100 MHz, respectively, with a Varian AS400 spectrometer and at 600 and 151 MHz, respectively, with a Bruker spectrometer, using TMS as an internal standard with CDCl₃ and DMSO-*d*₆. The coupling constants (*J* values) and chemical shifts (δ_{ppm}) were reported in Hertz (Hz) and parts per million (ppm) respectively. Multiplicities are reported as follows: s (singlet), d (doublet), t

(triplet), m (multiplet) and br (broadened). High-resolution mass spectra (HRMS) were recorded on an Agilent Q-TOF mass spectrometer with a Z-spray source using built-in software for analysis of the recorded data.

General procedure for the synthesis of Cbz-protected L-tryptophan and tryptamine

L-Tryptophan or tryptamine (29.4 mmol) was first dissolved in 1 M NaOH (60 mL) and stirred at 0 °C for 30 min. Then benzylchloroformate (32.3 mmol) and 1 M NaOH (30 mL) were simultaneously added to the solution in a dropwise fashion.³² The mixture was stirred for 12 hours at room temperature. The solution was acidified with 6 M HCl to pH 1–2 and extracted with EtOAc (3 × 200 mL). The combined organic layer was dried over anhydrous Na₂SO₄ and concentrated under reduced pressure. The product was used directly in the following reactions.

General procedure for the synthesis of benzyl-protected L-tryptophan

To a stirring solution of L-tryptophan (2.0 mmol) in toluene (50 mL) PTSA (4.8 mmol) was added. Then benzylalcohol (23.08 mmol) was added in a dropwise fashion under stirring conditions and the reaction mixture was refluxed for 24 hours using a Dean–Stark apparatus. Excess toluene was removed under reduced pressure and the reaction mixture was diluted with water (50 mL). The compound was then extracted with EtOAc (3 × 100 mL). The combined organic layer was dried over anhydrous Na₂SO₄ and concentrated under reduced pressure. Column chromatography with silica gel and a gradient solvent system of MeOH to DCM (5–10%) yielded the target products (75%).

Synthesis of 2-acetylamino-3-(1*H*-indol-3-yl)propanoic acid

A solution of L-tryptophan (1.5 mmol) and acetic anhydride (3 mmol) in methanol (20 mL) was refluxed overnight and then concentrated under reduced pressure.⁵¹ The crude mixture was then diluted with water and extracted with ethyl acetate (3 × 30 mL). The combined organic layer was dried over anhydrous Na₂SO₄ followed by concentration under reduced pressure to obtain the *N*-acetylated product, which was directly used for the next step without further purification.

Synthesis of 2-benzoylamino-3-(1*H*-indol-3-yl)propanoic acid

To a stirred solution of L-tryptophan (1.5 mmol) in 2 mL water was added 2 mL of 1 M NaOH aqueous solution followed by the dropwise addition of benzoyl chloride (1.8 mmol) dissolved in 0.5 M NaOH (8 mL) and chloroform (8 mL) at 0 °C under continuous stirring. Then the whole solution was allowed to stir at room temperature for 12 hours.⁵² After completion of the reaction (monitored by TLC), the residual solvent was removed under reduced pressure and the reaction mixture was further acidified with 0.5 M HCl to pH 3–4. The aqueous layer was then extracted with ethyl acetate (3 × 30

mL) and the combined organic layer was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to obtain the desired solid product, which was directly used for the next step.

General procedure for the synthesis of 2-indolinone derivatives of L-tryptophan and tryptamine

The desired amount of substituted L-tryptophan or tryptamine (1.5 mmol) was first dissolved in acetic acid (1.5 mL) and then a mixture of DMSO and concentrated HCl (1:3 approx.) was slowly added under continuous stirring conditions.³² The resulting solution was then stirred at room temperature for another 1–2 hours. After completion of the reaction (monitored by TLC), the reaction mixture was diluted with water (8 mL) and further extracted with ethyl acetate (3×30 mL). The combined organic layer was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. Column chromatography with silica gel and a gradient solvent system of methanol to dichloromethane (5–20%) yielded the target products.

Synthesis of 2-amino-3-(1*H*-indol-3-yl)propanamide (10)

To a stirring solution of compound 3 (1.5 mmol) in DMF (10 mL) were added 1-hydroxybenzotriazole (1.5 mmol) and EDCI (1.65 mmol). The whole mixture was then stirred for 2 hours at room temperature under a N_2 atmosphere.³⁴ Then aqueous ammonia solution (0.5 mL, 7.0 mmol) was added dropwise under continuous stirring conditions and the resulting solution was allowed to stir for another 18 hours at room temperature. Then water (20 mL) was added and the solution was washed with EtOAc (3×10 mL). The organic phase was again washed with 5% NaHCO_3 solution, dried over anhydrous Na_2SO_4 , and concentrated under reduced pressure. The crude mixture was further hydrogenated (1 atmosphere pressure) in MeOH using a catalytic amount of Pd/C (10 mol%) to obtain the crude product 10. The pure compound was isolated using column chromatography with silica gel and a gradient solvent system of methanol to dichloromethane (10–35%).

General procedure for the synthesis of hydrazone and phenylimino derivatives of isatin

To a stirring solution of isatin (1 mmol) in ethanol were added an appropriate amount of the aromatic hydrazine or primary amine (1 mmol) and a catalytic amount of acetic acid.³⁵ The whole solution was refluxed for 0.5–4 hours under constant stirring. After completion of the reaction, the solvent was evaporated under reduced pressure followed by washing the crude product with water (3×10 mL) and dried under reduced pressure. Column chromatography with silica gel and a gradient solvent system of ethyl acetate to hexane (5–20%) yielded the target products. Recrystallization from ethanol also yielded the desired pure product.

General procedure for the synthesis of 2-indolinone derivatives, 21 and 25

To an ice cooled mixture of isatin (2.5 mmol) and acetone or acetyl acetone (2.5 mmol) was added 0.5 mL of piperidine under continuous stirring.³⁶ The whole mixture was stirred at 0 °C for 4 hours and then cold ethanol was added. The resulting solid was filtered and dried under reduced pressure. Column chromatography with silica gel and a gradient solvent system of ethyl acetate to hexane (5–20%) yielded the target products.

General procedure for the synthesis of 2-indolinone derivatives, 20 and 24

A solution of isatin (1 mmol) and diethyl malonate (1 mmol) or dimedone (1 mmol) in water was refluxed with a catalytic amount of piperidinium acetate for 3–4 hours at 100 °C.³⁶ The thus obtained solid was filtered, washed properly with water and dried in an oven. Further purification was performed by silica gel column chromatography using an ethyl acetate:hexane gradient solvent system (5–20%) or recrystallized from ethanol to obtain the desired pure product.

General procedure for the synthesis of 5-chloroisatin derivatives

The compounds were synthesized according to the reported procedure with slight modification.³⁷ The desired amount of 5-chloroisatin (1.1 mmol) was dissolved in acetone (10 mL) and then an appropriate amount of K_2CO_3 (1.3 mmol) was added under stirring conditions. The whole solution was then refluxed for 14 hours at 50 °C. After completion of the reaction (monitored by TLC), the reaction mixture of 3-hydroxy 5-chloroisatin was concentrated under reduced pressure and the residue obtained was triturated with diethyl ether resulting in the alcohol intermediate as a white solid, which was directly used for the next step.

To a stirring solution of 3-hydroxy 5-chloroisatin in ethanol (5 mL) were added dropwise concentrated HCl (approx. 1 mL) and a catalytic amount of acetic acid. Then the mixture was refluxed for 12 hours at 60 °C under continuous stirring.³⁷ After the reaction was complete (monitored by TLC), the mixture was concentrated and washed with saturated sodium bicarbonate solution. The aqueous layer was then extracted with ethyl acetate (3×30 mL) and the combined organic layer was further dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. Column chromatography with silica gel and a gradient solvent system of ethyl acetate to hexane (5–25%) yielded the target products.

Characterization of the synthesized compounds

2-Amino-3-(2-oxoindolin-3-yl)propanoic acid (1). As pink solid (45% yield; rotamers, hygroscopic); ^1H NMR (600 MHz, CDCl_3 + $\text{DMSO}-d_6$) δ_{ppm} 10.46 (s, 1H), 8.82 (br, s, 1H), 7.47–

7.44 (m, 1H), 7.18 (d, 1H, $J = 12$ Hz), 7.13 (d, 1H, $J = 6$), 7.09–7.08 (m, 1H), 6.92–6.88 (m, 1H), 6.83–6.80 (m, 1H), 3.75–3.73 (m, 1H), 3.54–3.52 (m, 1H), 1.93–1.89 (m, 2H); ^{13}C NMR (100 MHz, $\text{CDCl}_3 + \text{DMSO-}d_6$) δ_{ppm} 179.8, (179.5), 169.8, 141.5, (141.4), 127.8, (127.7), 123.3, (123.2), 121.7, 109.8, (109.7), 51.3, (50.6), 43.5, (42.0), 30.2, (29.8); HRMS [ESI] calcd. for $\text{C}_{11}\text{H}_{12}\text{N}_2\text{O}_3$ $[\text{M} + \text{H}]^+$ 221.0921, found 221.0920.

3-(2-Aminoethyl)indolin-2-one (2). As light brown solid (70% yield; rotamers, mp: 245–247 °C); ^1H NMR (400 MHz, $\text{CDCl}_3 + \text{DMSO-}d_6$) δ_{ppm} 10.41 (br s, 1H), 8.38 (br s, 2H), 7.24–7.16 (m, 2H), 7.00–6.96 (m, 1H), 6.90 (d, 1H, $J = 8$ Hz), 3.56–3.53 (m, 1H), 3.11–3.06 (m, 2H), 2.38–2.29 (m, 1H), 2.24–2.14 (m, 1H); ^{13}C NMR (151 MHz, $\text{CDCl}_3 + \text{DMSO-}d_6$) δ_{ppm} 178.3, (178.2), 141.6, (141.5), 127.5, (127.4), 127.2, 122.9, (121.1), 121.0, 109.2, (109.1), 43.0, (42.9), 36.4, 27.1, (27.0); HRMS [ESI] calcd. for $\text{C}_{10}\text{H}_{12}\text{N}_2\text{O}$ $[\text{M} + \text{H}]^+$ 177.1022, found 177.1026.

2-(((Benzyloxy)carbonyl)amino)-3-(2-oxoindolin-3-yl)propanoic acid (3). As light brown semi-solid (70% yield; rotamers); ^1H NMR (600 MHz, $\text{CDCl}_3 + \text{DMSO-}d_6$) δ_{ppm} 9.48 (br s, 1H), 7.33–7.32 (m, 2H), 7.25–7.10 (m, 5H), 6.97–6.92 (m, 1H), 6.80 (d, 2H, $J = 6$ Hz), 5.04 (s, 2H), 4.59 (t, 1H), 3.48–3.34 (m, 1H), 2.36–2.31 (m, 2H); ^{13}C NMR (151 MHz, $\text{CDCl}_3 + \text{DMSO-}d_6$) δ_{ppm} 180.8, (180.6), 174.1, (174.0), 156.9, (156.5), 141.6, (141.5), 136.5, (136.3), 129.1, 128.5, (128.4), 128.3, 128.1, (128.0), 127.9, 124.7, 124.1, 122.8, (122.7), 110.2, 67.1, (66.9), 52.1, 40.3, 33.2, (32.0); HRMS [ESI] calcd. for $\text{C}_{19}\text{H}_{18}\text{N}_2\text{O}_5$ $[\text{M} + \text{H}]^+$ 355.1288, found 355.1289.

2-((Tert-butoxycarbonyl)amino)-3-(2-oxoindolin-3-yl)propanoic acid (4). As brown semisolid (68% yield; rotamers); ^1H NMR (400 MHz, $\text{CDCl}_3 + \text{CD}_3\text{OD}$) δ_{ppm} 7.61–7.59 (m, 1H), 7.13–7.09 (m, 1H), 6.51–6.45 (m, 2H), 4.28 (s, 1H), 3.61–3.43 (m, 1H), 2.92–2.91 (m, 2H), 1.27 (s, 9H); ^{13}C NMR (151 MHz, $\text{CDCl}_3 + \text{CD}_3\text{OD}$) δ_{ppm} 202.0, (201.9), 156.3, (156.3), 150.7, 134.8, 131.5, (131.4), 117.7, (117.5), 115.9, (115.8), 79.6, 52.2, 42.2, 29.7, 28.3; HRMS [ESI] calcd. for $\text{C}_{16}\text{H}_{20}\text{N}_2\text{O}_5$ $[\text{M} + \text{H}]^+$ 321.1445, found 321.1506.

2-Benzamido-3-(2-oxoindolin-3-yl)propanoic acid (isomeric mixture of 5). As yellow semi-solid (60% yield; rotamers, mp: 162–165 °C); ^1H NMR (400 MHz, $\text{CDCl}_3 + \text{DMSO-}d_6$) δ_{ppm} 10.15 (s, 1H), 10.07 (br s, 1H), 8.81–8.80 (m, 1H), 8.43 (br s, 1H), 7.91–7.84 (m, 2H), 7.47–7.33 (m, 3H), 7.20–7.09 (m, 1H), 6.97–6.91 (m, 1H), 6.84–6.80 (m, 1H), 4.96–4.90 (m, 1H), 4.82–4.78 (m, 1H), 2.61–2.51 (m, 1H), 2.40–2.24 (m, 1H); ^{13}C NMR (151 MHz, $\text{CDCl}_3 + \text{DMSO-}d_6$) δ_{ppm} 180.4, (179.8), 173.3, (173.2), 167.3, (166.8), 141.9, 133.6, (133.5), 131.3, (131.2), 128.0, 127.9, (127.7), 127.2, 124.2, (123.5), 122.0, (121.9), 109.8, (109.7), 51.2, 43.3, (43.1), 32.3, (31.3); HRMS [ESI] calcd. for $\text{C}_{18}\text{H}_{16}\text{N}_2\text{O}_4$ $[\text{M} + \text{H}]^+$ 325.1183, found 325.1155.

2-Acetamido-3-(2-oxoindolin-3-yl)propanoic acid (isomeric mixture of 6). As brown semi-solid (70% yield; rotamers); ^1H NMR (400 MHz, $\text{CDCl}_3 + \text{CD}_3\text{OD}$) δ_{ppm} 9.28 (br s, 1H), 6.59–6.55 (m, 1H), 6.49–6.40 (m, 1H), 6.26–6.22 (m, 1H), 6.16–6.13 (m, 1H), 4.07–4.02 (m, 1H), 3.89–3.86 (m, 1H), 1.89 (s, 3H), 1.69–1.61 (m, 1H), 1.54–1.48 (m, 1H); ^{13}C NMR (151 MHz, $\text{CDCl}_3 + \text{CD}_3\text{OD}$) δ_{ppm} 180.3, (180.2), 172.2, (171.9), 141.7,

(141.6), 128.3, (128.1), 124.2, 123.7, 110.1, (110.0), 50.3, (50.1), 39.8, 31.9, (31.1), 22.0, (21.9); HRMS (ESI) calcd. for $\text{C}_{13}\text{H}_{14}\text{N}_2\text{O}_4$ $[\text{M} + \text{H}]^+$ 263.1026, found 263.1012.

Benzyl 2-(2-oxoindolin-3-yl)ethylcarbamate (7). As light brown solid (70% yield; rotamers, mp: 161–163 °C); ^1H NMR (400 MHz, $\text{CDCl}_3 + \text{DMSO-}d_6$) δ_{ppm} 9.65 (br s, 1H), 7.39–7.25 (m, 5H), 7.20–7.15 (m, 1H), 7.01–6.95 (m, 1H), 6.87–6.86 (m, 2H), 5.95 (br s, 1H), 5.07 (s, 2H), 3.43–3.38 (m, 1H), 2.74–2.65 (m, 2H), 2.12–2.09 (m, 2H); ^{13}C NMR (151 MHz, $\text{CDCl}_3 + \text{DMSO-}d_6$) δ_{ppm} 179.2, (179.1), 155.9, 141.6, 136.2, 128.7, 127.9, (127.4), 127.3, 123.5, (123.4), 121.4, 109.2, 65.8, 43.3, 37.8, 30.0; HRMS [ESI] calcd. for $\text{C}_{18}\text{H}_{18}\text{N}_2\text{O}_3$ $[\text{M} + \text{H}]^+$ 311.1390, found 311.1398.

Benzyl 2-amino-3-(2-oxoindolin-3-yl)propanoate (8). As yellow semi-solid (55% yield; rotamers); ^1H NMR (400 MHz, $\text{CDCl}_3 + \text{CD}_3\text{OD}$) δ_{ppm} 7.57 (d, 2H, $J = 8$ Hz), 7.37 (d, 1H, $J = 8$ Hz), 7.27–6.92 (m, 6H), 5.05 (s, 2H), 4.03 (t, 1H), 3.23–3.18 (m, 1H), 2.19 (s, 2H); ^{13}C NMR (151 MHz, $\text{CDCl}_3 + \text{CD}_3\text{OD}$) δ_{ppm} 174.6, (174.6), 170.6, 140.4, (141.3), 136.5, 128.7, 128.5, 128.3, 125.5, 124.4, 121.7, 119.1, 117.7, 111.5, (111.4), 67.7, 64.1, 53.5, 39.7; HRMS [ESI] calcd. for $\text{C}_{18}\text{H}_{18}\text{N}_2\text{O}_3$ $[\text{M} + \text{H}]^+$ 311.1390, found 311.1395.

Benzyl 2-(((benzyloxy)carbonyl)amino)-3-(2-oxoindolin-3-yl)propanoate (9). As light brown oil (55% yield; rotamers); ^1H NMR (600 MHz, $\text{CDCl}_3 + \text{DMSO-}d_6$) δ_{ppm} 9.18 (br s, 1H), 7.33–7.10 (m, 10H), 6.99–6.94 (m, 2H), 6.84 (d, 1H, $J = 12$ Hz), 6.40 (d, 1H, $J = 12$ Hz), 6.24 (d, 1H, $J = 12$ Hz), 5.06 (s, 4H), 4.66–4.64 (m, 1H), 3.53–3.51 (m, 1H), 2.38–2.31 (m, 1H); ^{13}C NMR (151 MHz, $\text{CDCl}_3 + \text{DMSO-}d_6$) δ_{ppm} 175.8, 174.8, (174.7), 156.7, (156.5), 141.3, 136.5, (136.3), 128.7, (128.6), 128.5, (128.4), 128.3, (128.2), 124.6, (124.3), 123.1, 110.6, (110.5), 67.3, (67.1), 53.2, (51.9), 43.5, (43.0), 40.3, (40.2), 32.8, (31.9); HRMS [ESI] calcd. for $\text{C}_{26}\text{H}_{24}\text{N}_2\text{O}_5$ $[\text{M} + \text{H}]^+$ 445.1758, found 445.1762.

2-Amino-3-(2-oxoindolin-3-yl)propanamide (10). As yellow semi-solid (45% yield; rotamers); ^1H NMR (400 MHz, $\text{CDCl}_3 + \text{DMSO-}d_6$) δ_{ppm} 7.91–7.86 (m, 1H), 7.81–7.73 (m, 1H), 7.66–7.60 (m, 1H), 7.49–7.36 (m, 1H), 3.67–3.63 (m, 1H), 3.01–2.97 (m, 1H), 2.27–2.23 (m, 1H), 2.13–2.09 (m, 1H); ^{13}C NMR (151 MHz, $\text{CD}_3\text{OD} + \text{DMSO-}d_6$) δ_{ppm} 181.2, 180.1, 144.4, 133.0, (132.9), 128.2, 123.7, 118.7, (118.4), 111.8, (111.3), 52.9, 43.1, 37.1; HRMS [ESI] calcd. for $\text{C}_{11}\text{H}_{13}\text{N}_3\text{O}_2$ $[\text{M} + \text{NH}_4]^+$ (237.1346), found 237.0859.

3-(2-Phenylhydrazono)indolin-2-one (11). As yellow solid (85% yield; mp: 208–210 °C (lit. mp 214–216 °C)³²); ^1H NMR (600 MHz, $\text{CDCl}_3 + \text{DMSO-}d_6$) δ_{ppm} 12.69 (br s, 1H), 7.77 (br s, 1H), 7.64–7.63 (m, 1H), 7.38–7.34 (m, 4H), 7.23–7.20 (m, 1H), 7.10–7.08 (m, 1H), 7.06–7.04 (m, 1H), 6.90–6.88 (m, 1H); ^{13}C NMR (151 MHz, $\text{CDCl}_3 + \text{DMSO-}d_6$) δ_{ppm} 163.6, 142.7, 138.2, 129.6, 128.3, 126.9, 123.6, 122.9, 122.3, 119.4, 114.6, 110.4; HRMS [ESI] calcd. For $\text{C}_{14}\text{H}_{11}\text{N}_3\text{O}$ $[\text{M} + \text{H}]^+$ 238.0975, found 238.0977.

3-[2-(4-Chlorophenyl)hydrazono]indolin-2-one (12). As yellow solid (88% yield; mp: mp 262–264 °C (lit. mp 266–267 °C)³²); ^1H NMR (600 MHz, $\text{CDCl}_3 + \text{DMSO-}d_6$) δ_{ppm} 10.59 (br s, 1H), 7.58–7.56 (m, 1H), 7.37–7.33 (m, 4H), 7.25–7.22 (m,

1H), 7.07–7.05 (m, 1H), 6.94–6.93 (m, 1H); ^{13}C NMR (151 MHz, CDCl_3 + $\text{DMSO}-d_6$) δ_{ppm} 163.6, 140.9, 139.3, 128.8, 127.9, 126.9, 121.5, 121.0, 118.5, 114.7, 110.2; HRMS [ESI] calcd. for $\text{C}_{14}\text{H}_{10}\text{ClN}_3\text{O}$ $[\text{M} + \text{H}]^+$ 272.0585, found 272.0584.

3-[2-(3-Chlorophenyl)-hydrazono]indolin-2-one (13). As yellow solid (80% yield; mp: 237–239 °C (lit. mp 233–234 °C)³²); ^1H NMR (600 MHz, CDCl_3 + $\text{DMSO}-d_6$) δ_{ppm} 10.14 (br s, 1H), 7.44–7.43 (m, 1H), 7.11–7.04 (m, 3H), 6.98–6.97 (m, 1H), 6.90–6.88 (m, 1H), 6.82–6.80 (m, 1H), 6.76–6.75 (m, 1H); ^{13}C NMR (151 MHz, CDCl_3 + $\text{DMSO}-d_6$) δ_{ppm} 163.9, 135.0, 130.3, 128.9, 128.9, 128.5, 122.3, 122.0, 121.3, 119.0, 113.7, 112.3, 110.6; HRMS [ESI] calcd. for $\text{C}_{14}\text{H}_{10}\text{ClN}_3\text{O}$ $[\text{M} + \text{H}]^+$ 272.0585, found 272.0587.

3-[2-(3-Nitrophenyl)-hydrazono]indolin-2-one (14). As yellow solid (70% yield; mp: 263–265 °C (lit. mp 267–269 °C)³⁵); ^1H NMR (600 MHz, CDCl_3 + $\text{DMSO}-d_6$) δ_{ppm} 10.53 (br s, 1H), 8.20 (s, 1H), 7.83–7.82 (m, 1H), 7.64–7.60 (m, 1H), 7.52–7.49 (m, 2H), 7.24–7.23 (m, 1H), 7.08–7.06 (m, 1H), 6.94–6.93 (m, 1H); ^{13}C NMR (151 MHz, CDCl_3 + $\text{DMSO}-d_6$) δ_{ppm} 163.7, 149.0, 143.8, 140.1, 138.2, 130.1, 128.9, 122.0, 120.8, 119.4, 119.2, 116.4, 110.6, 108.1; HRMS [ESI] calcd. for $\text{C}_{14}\text{H}_{10}\text{N}_4\text{O}_3$ $[\text{M} + \text{H}]^+$ 283.0826, found 283.0833.

3-[2-(2-(Trifluoromethyl)phenyl)-hydrazono]indolin-2-one (15). As light yellow solid (65% yield; mp: 252–254 °C); ^1H NMR (600 MHz, CDCl_3 + $\text{DMSO}-d_6$) δ_{ppm} 8.04–8.02 (m, 1H), 7.66–7.58 (m, 2H), 7.44–7.39 (m, 2H), 7.27–7.26 (m, 1H), 7.11–7.10 (m, 1H), 6.94–6.92 (m, 1H); ^{13}C NMR (151 MHz, CDCl_3 + $\text{DMSO}-d_6$) δ_{ppm} 163.1, 140.1, 140.0, 132.7, 130.4, 128.6, 125.5, 121.5, 120.5, 118.8, 114.4, 113.9, 110.2; HRMS [ESI] calcd. for $\text{C}_{15}\text{H}_{10}\text{F}_3\text{N}_3\text{O}$ $[\text{M} + \text{H}]^+$ 306.0849, found 306.0850.

4-Methyl-N'-(2-oxoindolin-3-ylidene)benzenesulfonohydrazide (16). As yellow solid (65% yield; mp: 196–198 °C (lit. mp 188 °C)³⁶); ^1H NMR (600 MHz, CDCl_3 + $\text{DMSO}-d_6$) δ_{ppm} 8.10–8.09 (m, 2H), 8.01–7.99 (m, 2H), 7.58 (br s, 1H), 7.50–7.42 (m, 2H), 7.20–7.17 (m, 1H), 7.03–7.01 (m, 1H), 2.57 (s, 3H); ^{13}C NMR (151 MHz, CDCl_3 + $\text{DMSO}-d_6$) δ_{ppm} 148.1, 138.8, 135.0, 133.3, 133.1, 131.8, 131.2, 126.3, 125.8, 124.7, 123.2, 114.5, 24.9; HRMS [ESI] calcd. for $\text{C}_{15}\text{H}_{13}\text{N}_3\text{O}_3\text{S}$ $[\text{M} + \text{H}]^+$ 316.0750, found 316.0751.

3-(Phenylimino)indolin-2-one (17). As yellow solid (75% yield; mp: 230–232 °C); ^1H NMR (600 MHz, CDCl_3 + $\text{DMSO}-d_6$) δ_{ppm} 10.26 (br s, 1H), 7.36–7.34 (m, 1H), 7.30–7.29 (m, 1H), 7.20–7.16 (m, 2H), 7.93–7.92 (m, 2H), 6.82–6.80 (m, 1H), 6.61–6.60 (m, 1H), 6.48–6.47 (m, 1H); ^{13}C NMR (151 MHz, CDCl_3 + $\text{DMSO}-d_6$) δ_{ppm} 164.7, 155.2, 150.4, 146.8, 134.1, 129.3, 126.1, 125.1, 122.0, 117.7, 116.0, 111.6; HRMS [ESI] calcd. for $\text{C}_{14}\text{H}_{10}\text{N}_2\text{O}$ $[\text{M} + \text{H}]^+$ 223.0866, found 223.0870.

3-[(4-Chlorophenyl)-imino]indolin-2-one (18). As yellow solid (70% yield; mp: 237–239 °C); ^1H NMR (600 MHz, CDCl_3 + $\text{DMSO}-d_6$) δ_{ppm} 10.32 (br s, 1H), 7.26–7.24 (m, 1H), 7.16–7.12 (m, 1H), 6.83–6.81 (m, 2H), 6.76–6.75 (m, 1H), 6.59–6.57 (m, 1H), 6.50–6.49 (m, 2H); ^{13}C NMR (151 MHz, CDCl_3 + $\text{DMSO}-d_6$) δ_{ppm} 164.4, 155.7, 148.7, 147.1, 134.4, 129.4, 126.0, 122.0, 120.6, 119.4, 115.8, 111.8; HRMS [ESI] calcd. for $\text{C}_{14}\text{H}_9\text{ClN}_2\text{O}$ $[\text{M} + \text{H}]^+$ 257.0476, found 257.0476.

3-[(2-Bromophenyl)-imino]indolin-2-one (19). As yellow solid (45% yield; mp: 237–239 °C); ^1H NMR (600 MHz, CDCl_3 + $\text{DMSO}-d_6$) δ_{ppm} 10.51 (br s, 1H), 7.69–7.67 (m, 1H), 7.39–7.36 (m, 1H), 7.34–7.31 (m, 1H), 7.13–7.10 (m, 1H), 6.98–6.95 (m, 2H), 6.77–6.74 (m, 1H), 6.50–6.49 (m, 1H); ^{13}C NMR (151 MHz, CDCl_3 + $\text{DMSO}-d_6$) δ_{ppm} 165.3, 156.4, 148.9, 146.0, 135.2, 133.7, 128.7, 126.6, 123.3, 118.8, 116.5, 112.3; HRMS [ESI] calcd. for $\text{C}_{14}\text{H}_9\text{BrN}_2\text{O}$ $[\text{M} + \text{H}]^+$ 300.9971, found 300.9974.

Diethyl 2-(2-oxoindolin-3-ylidene)malonate (20). As red semi-solid (60% yield); ^1H NMR (600 MHz, CDCl_3 + $\text{DMSO}-d_6$) δ_{ppm} 8.81 (br s, 1H), 8.33 (d, 1H, $J = 12$ Hz), 7.30 (t, 1H), 6.70 (t, 1H), 6.82 (d, 1H, $J = 6$ Hz), 4.44–4.35 (m, 4H), 1.38–1.24 (m, 6H); ^{13}C NMR (151 MHz, CDCl_3 + $\text{DMSO}-d_6$) δ_{ppm} 168.0, 165.6, 163.1, 143.8, 135.0, 133.4, 129.7, 129.2, 123.0, 119.9, 110.5, 62.5, 14.1; HRMS [ESI] calcd. for $\text{C}_{15}\text{H}_{15}\text{NO}_5$ $[\text{M} + \text{H}]^+$ 290.1023, found 290.1025.

3-(2-Oxopropylidene)indole-2-one (21). As red solid (88% yield; mp: 172–174 °C); ^1H NMR (400 MHz, CDCl_3) δ_{ppm} 8.44 (d, 1H, $J = 8$ Hz), 7.27 (t, 1H), 7.20 (s, 1H), 6.97 (t, 1H), 6.79 (d, 1H, $J = 8$ Hz), 2.42 (s, 3H); ^{13}C NMR (100 MHz, CDCl_3) δ_{ppm} 198.7, 170.0, 143.6, 135.7, 133.3, 128.8, 128.0, 123.2, 120.9, 110.3, 32.6; HRMS [ESI] calcd. for $\text{C}_{11}\text{H}_9\text{NO}_2$ $[\text{M} + \text{H}]^+$ 188.0706, found 188.0708.

5-Chloro-3-(2-oxopropylidene)indole-2-one (22). As red solid (75% yield; mp: 183–185 °C); ^1H NMR (400 MHz, CDCl_3) δ_{ppm} 8.59 (d, 1H, $J = 4$ Hz), 7.40–7.38 (m, 1H), 6.89 (s, 1H), 6.87 (s, 1H), 2.60 (s, 3H); ^{13}C NMR (100 MHz, CDCl_3 + $\text{CD}_3\text{-OD}$) δ_{ppm} 198.8, 169.8, 142.7, 135.3, 132.8, 128.9, 128.4, 121.9, 111.3, 32.4; HRMS [ESI] calcd. for $\text{C}_{11}\text{H}_8\text{ClNO}_2$ $[\text{M} + \text{H}]^+$ 222.0316, found 222.0322.

1-(5-Chloro-2-oxoindolin-3-ylidene)pentane-2,4-dione (23). As brown solid (60% yield; mp: 128–130 °C); ^1H NMR (400 MHz, CDCl_3 + $\text{DMSO}-d_6$) δ_{ppm} 9.45 (br s, 1H), 7.23 (s, 1H), 7.12 (d, 1H, $J = 8$ Hz), 6.76–6.73 (m, 1H), 5.41 (s, 1H), 2.88 (s, 1H), 2.09 (s, 1H), 1.93 (s, 3H); ^{13}C NMR (100 MHz, CDCl_3 + $\text{DMSO}-d_6$) δ_{ppm} 191.8, 189.2, 178.6, 140.0, 132.1, 129.6, 127.8, 124.9, 111.6, 101.5, 74.9, 45.6, 24.1; HRMS [ESI] calcd. for $\text{C}_{13}\text{H}_{10}\text{ClNO}_3$ $[\text{M} + \text{H}]^+$ 282.0528, found 282.0398.

2-(3-Hydroxy-2-oxoindolin-3-yl)cyclohexane-1,3-dione (24). As light brown solid (55% yield; mp: 296–298 °C); ^1H NMR (600 MHz, CDCl_3 + $\text{DMSO}-d_6$) δ_{ppm} 10.44 (br s, 1H), 9.01 (s, 1H), 7.16–7.14 (m, 1H), 6.93–6.88 (m, 2H), 6.85–6.84 (m, 1H), 3.48 (s, 1H), 2.39–2.10 (m, 4H), 1.98–1.96 (m, 2H); ^{13}C NMR (151 MHz, CDCl_3 + $\text{DMSO}-d_6$) δ_{ppm} 202.5, 170.2, 144.1, 121.7, 120.4, 112.8, 109.8, 100.2, 60.3, 46.7, 36.0, 20.1; HRMS [ESI] calcd. for $\text{C}_{14}\text{H}_{13}\text{NO}_4$ $[\text{M} + \text{H}]^+$ 260.0917, found 260.1440.

1-(3-Hydroxy-2-oxoindolin-3-yl)pentane-2,4-dione (25). As brown semi-solid (55% yield; rotamers); ^1H NMR (600 MHz, CDCl_3 + $\text{DMSO}-d_6$) δ_{ppm} 7.44–7.42 (m, 1H), 7.30–7.26 (m, 1H), 6.67–6.62 (m, 2H), 6.36 (br s, 1H), 3.67 (s, 2H), 3.28–3.26 (m, 2H), 1.69–1.65 (m, 3H); ^{13}C NMR (151 MHz, CDCl_3 + $\text{DMSO}-d_6$) δ_{ppm} 194.5, 165.8, 151.7, 136.1, (136.0), 133.4, (133.2), 117.3, (117.2), 116.5, (116.3), 114.4, 47.3, (47.2), 42.2, (42.0), 26.3, 25.6, (24.5); HRMS [ESI] calcd. for $\text{C}_{13}\text{H}_{13}\text{NO}_4$ $[\text{M} + \text{Na}]^+$ 271.0769 found 271.0906.

5-Chloro-3-hydroxy-3-(2-oxopropyl)indolin-2-one (26). As white solid (75% yield; mp: 188–190); ^1H NMR (400 MHz, CDCl_3 , $\text{DMSO}-d_6$) δ_{ppm} 9.58 (br, s, 1H), 7.32 (s, 1H), 7.23 (d, 1H, $J = 8$ Hz), 6.87–6.84 (m, 1H), 5.49 (s, 1H), 3.29–3.11 (m, 2H), 2.19 (s, 3H); ^{13}C NMR (100 MHz, $\text{CDCl}_3 + \text{CD}_3\text{OD}$) δ_{ppm} 205.8, 178.9, 140.4, 133.2, 129.7, 127.1, 124.0, 111.1, 73.0, 49.4, 29.1; HRMS [ESI] calcd. for $\text{C}_{11}\text{H}_{10}\text{ClNO}_3$ [$\text{M} + \text{H}$] $^+$ 240.0422, found 240.0420.

1-(5-Chloro-3-hydroxy-2-oxoindolin-3-yl)pentane-2,4-dione (27). As yellow semi-solid (40% yield; mp: 142–144 °C); ^1H NMR (600 MHz, CDCl_3) δ_{ppm} 7.41 (s, 1H), 7.25–7.24 (m, 1H), 6.65–6.62 (m, 1H), 6.36 (br, s, 1H), 3.69 (s, 2H), 3.28 (s, 2H), 1.69 (s, 3H); ^{13}C NMR (151 MHz, CDCl_3) δ_{ppm} 193.5, 165.1, 150.2, 136.2, 132.0, 120.8, 118.9, 115.1, 47.4, 42.4, 26.4, 24.6; HRMS [ESI] calcd. for $\text{C}_{13}\text{H}_{12}\text{ClNO}_4$ [$\text{M} + \text{H}$] $^+$ 282.0528 found 282.0398.

Purification of the compounds by HPLC analysis

All synthesized compounds were further purified by analytical-HPLC analyses (with a purity level of ≥ 94 –95%) before performing *in vitro* enzyme activity, cellular activity, cell viability, and other assays. A Waters 600 HPLC system with an Ascentis® Express C18 2.7 μm analytical column (Sigma) at a flow rate of 0.5 mL min^{-1} was used for the purification of the compounds. All the compounds (~ 1 mg) were dissolved in MeOH (1 mL) for HPLC analyses. All the compounds exhibited a strong absorption peak at 280 nm. Hence, the HPLC analyses were performed using a UV-detector at 280 nm. During each injection 20 μL of the compound solution was used and fractions were collected. This step was repeated more than 10-times to obtain sufficient amounts of the pure compounds. The total run time was 10 min. All the collected fractions for each compound were dried under reduced pressure and verified by HRMS analyses. The mobile phase for HPLC measurements was 60% MeOH and 40% H_2O (isocratic mode).

Expression and purification of recombinant human IDO1 and TDO enzymes

The cDNAs of human IDO1 (in the vector pQE30) and TDO (in the vector pET28a) were used for expression of recombinant human IDO1 and TDO enzymes. The human cDNAs of IDO1 and TDO enzymes were a generous gift from Professor Emma Raven (University of Leicester). Both enzymes were purified according the reported procedures with minor modifications.^{12,38,45} Briefly, during the protein expression isopropyl β -D-1-thiogalactopyranoside (IPTG), hemin and phenylmethylsulfonyl fluoride (PMSF) were added to the culture at final concentrations of 1 mM, 7 μM and 1 mM, respectively. The cells were grown for 3 hours at 25 °C and 120 rpm. After this incubation period, ethylenediaminetetraacetic acid (EDTA) was added to the culture at a final concentration of 1 mM and cells were grown for another 12 hours. The cells were collected by centrifugation at 5000 rpm for 10 minutes at 4 °C. The cell pellet was re-

suspended in 20 mL of ice-cold phosphate-buffered saline (PBS) containing 1 mM PMSF and 1 mM EDTA and then centrifuged at 15 000 rpm for 15 minutes at 4 °C. The pellet was stored at -80 °C. The washed pellet, obtained as described above, was re-suspended in 20 mL of ice-cold 50 mM potassium phosphate buffer (KPB) at pH 6.5 containing 300 mM potassium chloride (KCl), 10 mM imidazole, 10 mM magnesium chloride (MgCl_2), protease inhibitors (complete EDTA-free) and DNase (< 1 mg). The cell lysate was centrifuged at 20 000 rpm for 30 minutes at 4 °C, followed by filtering the supernatant through a 0.22 μm filter. After that, 1 mL of nickel-nitrilotriacetic acid resin (Qiagen) was added to the clear supernatant and the solution was incubated on ice for 2 hours at 80 rpm. Then, the mixture was poured into the column filled with 50 mM KPB at pH 6.5 containing 300 mM KCl and 10 mM imidazole. For the IDO1 enzyme, the column was sequentially washed with 10 mL of KPB at pH 6.5 containing 300 mM KCl and 20 and 30 mM imidazole, respectively, to remove the non-specifically bound protein. The IDO1 protein was eluted with 50 mM KPB at pH 6.5 containing 300 mM KCl and 190 mM imidazole. For the TDO enzyme, the column was sequentially washed with 10 mL of KPB at pH 8.0 containing 300 mM KCl and 20 and 30 mM imidazole, respectively, to remove the non-specifically bound protein. The TDO protein was eluted with 50 mM KPB at pH 8.0 containing 300 mM KCl and 190 mM imidazole. Both IDO1 and TDO proteins were then buffer exchanged by 50 mM Tris-buffer at pH 7.4 and 50 mM Tris-buffer at pH 8.0, respectively, using a Sephadex-G25 column. The purity of the enzyme was confirmed by Coomassie-blue staining SDS-PAGE analysis and it showed an 85–90% purity. The ratio of absorbance of the purified IDO1 and TDO enzymes at 404 nm to that at 280 nm was around 1.3 and 1.2, respectively.

IDO1 and TDO inhibition assay by spectrometric method

Both IDO1 and TDO inhibition assays were performed according to the earlier reported procedures.^{2,12,38,45} The solubility of the compounds in water was either moderate or poor. Hence, stock solutions of the compounds were prepared by first dissolving them in DMSO and then diluted with buffer. In the assay system the minimum and maximum amounts of DMSO were 0.02% and 2%, respectively. The standard reaction mixture (500 μL) contained KPB (100 mM, pH 6.5 for IDO1 enzyme and 50 mM, pH 8 for TDO enzyme), sodium ascorbate (20 mM), methylene blue (10 μM), catalase (240 nM, from brovin liver), L-Trp (150 μM), purified enzyme (40 nM for IDO1 and 25 nM for TDO), DMSO (0.05%, v/v), Triton-X 100 (0.01%, v/v) and inhibitors. The reaction was quenched using 100 μL of 30% (w/v) trichloroacetic acid. The amount of formed kynurenine was quantified using 2% (w/v) *p*-dimethylaminobenzaldehyde (*p*DMAB) in acetic acid. The absorbance of the reaction mixture was recorded at 480 nm. All these experiments were repeated three times for each compound.

Binding analysis by spectroscopic measurement

The absorption spectra were recorded at room temperature using a Perkin Elmer Lambda-25 UV-Vis spectrophotometer. All the measurements were performed in 100 mM Tris buffer pH 7.4 with an IDO1 enzyme concentration of 5 μM and a compound concentration of 50 μM . The deoxy-reaction system was prepared by injecting sodium dithionite (~10-fold excess) into the samples pre-purged with N_2 gas.^{12,13,45}

Determination of modes of enzyme inhibition by the compounds

The IDO1 enzyme inhibition mode of the selected compounds was measured according to the reported method.^{12,45,53} The IDO1 enzyme kinetics was determined using 50, 100 and 150 μM of L-Trp and 200, 500, 1000 and 1500 nM of inhibitor concentrations. The amount of generated N-formylkynurenine was monitored at different time intervals. The mode of inhibition was determined from the plot of $1/V$ vs. $1/[S]$, where $[S]$ and V represent the L-tryptophan concentration and initial rate of enzyme catalysis, respectively.

Molecular docking analysis

Molecular docking analyses of the interactions of the compounds with the IDO1 enzyme (PDB code: 2D0T) were performed using MoleGro Virtual Docker version 6.0 (Molegro ApS, Aarhus, Denmark).^{12,45,48,54–56} To generate the apo-protein, the ligands were first removed from the co-crystal structures and then were processed using energy minimization. The energy minimized three-dimensional structure of the ligands was prepared by using the GlycoBioChem PRODRG2 server (<http://davapc1.bioch.dundee.ac.uk/prodrg/>). The occupied position of the ligand (in the crystal structure) was used as the center of docking site (radius: 12 Å; and center: $x = 61$, $y = 51$, $z = 19$). Other parameters were set to default during docking analyses. In each docking run, two hundred docked structures were generated from an individual ligand. Energetically favored docked conformations were evaluated based on the moledock and re-rank scores (docking score-based on energy function such as a force field with repulsive and attractive van der Waals terms and electrostatic terms). The docking poses were exported and examined using PyMOL software (The PyMol Molecular Graphics System, Version 1.0r1, Schrödinger, LLC).

IDO1 and TDO inhibition assay by HPLC analysis

The enzymatic reaction (100 μL) was performed in 100 mM potassium phosphate buffer at pH 6.5 using sodium-ascorbate (20 mM), catalase (240 nM), methylene blue (10 μM), purified recombinant IDO1 (41 nM), L-Trp (150 μM), DMSO (0.05%, v/v) and Triton-X 100 (0.01%, v/v). First, the assay was performed using the inhibitors at different concentrations of 0.5 to 1.5 μM of the compounds. The reaction was

performed at 37 °C for 1 h and quenched by addition of 30% (w/v) trichloroacetic acid (20 μL). The reaction mixture was incubated at 50 °C for 30 minutes and then centrifuged at 10 000 rpm for 10 minutes. Then, 20 μL of supernatant from each reaction mixture was used for HPLC analyses. The mobile phase for HPLC measurements was 50% sodium citrate buffer (40 mM, pH 2.25) and 50% methanol with 400 μM SDS. The rate of flow through the Ascentis® Express C18, 2.7 μm HPLC column was 0.5 mL min^{-1} , and kynurenine was detected at a wavelength of 365 nm. Similar HPLC analyses were performed using pure kynurenine and a standard curve was prepared. The IC_{50} values of the compounds were calculated from this standard curve. Similarly, TDO enzyme inhibition activity assay was performed for these selected compounds.

Cellular activity assay

Breast cancer cells, MDA-MB-231, were selected for the *in vitro* cellular assay.^{12,45} 50 000 cells were seeded in each well of a 24-well plate in DMEM F12 complete media and allowed to adhere overnight. First, the cells were treated with different concentrations of human IFN- γ (from 5–1000 ng mL^{-1}) in complete media for a period of 48 h. Following this, 150 μM tryptophan was added and the cells were treated for an additional period of 5 h. For the post treatment phase, the cells were washed with sterile cell-culture grade PBS and were trypsinized and centrifuged at 1000 rpm. The cell pellet was dissolved again in sterile PBS and centrifuged at 1000 rpm as a period of washing. The pellet was hypotonically lysed in 10 mM HEPES buffer by passing through a sterile syringe 10 times. This lysate was used for the standard IDO1 assay as mentioned earlier. The results showed that 20 ng mL^{-1} of human IFN- γ is enough to show activity under the cellular conditions.¹² Therefore, all cell experiments were performed using this concentration of IFN- γ . After that, the cells were treated with human IFN- γ (20 ng mL^{-1}) in complete media for a period of 48 h. This treatment is reported to allow over-expression of IDO1 enzymes in MDA-MB-231 cells.^{12,46} Next, the cells were treated with appropriate concentrations of the compounds (20 nM to 1 mM) for a period of 4 h. Following this, 150 μM tryptophan was added and treated for an additional period of 5 h. Cells stimulated with IFN- γ alone served as negative control while cells stimulated with IFN- γ and then with 150 μM tryptophan served as positive control. For the post treatment phase, the cells were washed with sterile cell-culture grade PBS and were trypsinized and centrifuged at 1000 rpm. The cell pellet was dissolved again in sterile PBS and centrifuged at 1000 rpm as a period of washing. The pellet was hypotonically lysed in 10 mM HEPES buffer by passing through a sterile syringe 10 times. This lysate was used for the standard IDO1 assay as mentioned earlier.^{12,38,43} IC_{50} values were determined for each inhibitor accordingly.

Cell viability analysis

MDA-MB-231 breast cancer cells, A549 lung cancer cells and HeLa cervical cancer cells were cultured in DMEM/F12 media. J774A.1 macrophage cells were cultured in DMEM high glucose media. Both the media were supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin solution. Cells were maintained at 37 °C in a humidified 5% CO₂ incubator. 10 000 cells (of MDA-MB-231, A549, HeLa and J774A.1 cells) were seeded overnight in a 96 well plate with a total volume of 0.2 mL in their respective complete media. After 12 hours cells were washed twice with cell culture grade phosphate buffer saline (PBS) and were incubated with the IDO1 inhibitors (at IC₅₀ and 2 × IC₅₀ values respectively) in 0.2 mL of DMEM/F12 serum-free medium (incomplete medium) for 24 h and 48 h.^{12,45,57} Cells were also treated with mitomycin-C at 50 μM and 100 μM concentrations prepared in serum free media, which served as positive control. Cells treated with incomplete medium alone were considered as 100% viable (served as negative control). The dye MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was used to measure cellular viability. After the treatment period, the cells were washed twice with PBS and taken for morphological analysis *via* the Cytell imaging system (GE Healthcare). Images were collected at 10× magnification. After imaging, each well was incubated with 100 μL of MTT (0.5 mg mL⁻¹ in PBS) for 4 h at 37 °C with 5% CO₂. Then, the MTT solution was removed and the formazan crystals were dissolved in 100 μL cell culture grade DMSO. The absorbance was determined using a spectrophotometer (SpectraMax M2) at 570 nm and 660 nm (to subtract scattering effects of crystals). MDA-MB-231 and J774A.1 cell lines were procured from the National Cell Culture Facility, Central Drug Research Institute, Lucknow, India. A549 and HeLa Cell lines were procured from the National Center for Cell Science, Pune, India.

Conflict of interest

The authors declare no competing interests.

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