Fused Heterocyclic Compounds as Potent Indoleamine-2,3-dioxygenase 1 Inhibitors

Subhankar Panda,^{†,§} Ashalata Roy,^{†,§} Suman Jyoti Deka,[‡] Vishal Trivedi,[‡] and Debasis Manna^{*,†}[†]Department of Chemistry and [‡]Department of Bioscience and Bioengineering, Indian Institute of Technology, Guwahati, Assam 781039, India

S Supporting Information

ABSTRACT: Uncontrolled metabolism of L-tryptophan (L-Trp) in the immune system has been recognized as a critical cellular process in immune tolerance. Indoleamine 2,3-dioxygenase 1 (IDO1) enzyme plays an important role in the metabolism of a local L-Trp through the kynurenine pathway in the immune systems. In this regard, IDO1 has emerged as a therapeutic target for the treatment of diseases that are associated with immune suppression like chronic infections, cancer, and others. In this study, we synthesized a series of pyridopyrimidine, pyrazolopyranopyrimidine, and dipyrzolopyran derivatives. Further lead optimizations directed to the identification of potent compounds, **4j** and **4l** (IC_{50} = 260 and 151 nM, respectively). These compounds also exhibited IDO1 inhibitory activities in the low nanomolar range in MDA-MB-231 cells with very low cytotoxicity. Stronger selectivity for the IDO1 enzyme (>300-fold) over tryptophan 2,3-dioxygenase (TDO) enzyme was also observed for these compounds. Hence, these fused heterocyclic compounds are attractive candidates for the advanced study of IDO1-dependent cellular function and immunotherapeutic applications.

KEYWORDS: Indoleamine 2,3-dioxygenase 1 inhibition, fused heterocyclic compounds, halogen substituents, low IC_{50} and EC_{50} values, low cytotoxicity

Immunotherapy is currently considered as one of the most promising approaches in the battle against cancer.^{1–3} Recent accomplishment with the immune checkpoint inhibitors against a wide range of cancers has made cancer immunotherapy one of the most exciting developments. It has been also shown that cancer immunotherapy and traditional chemotherapy or radiotherapy could also be benefited from combinatorial strategies against tumor-induced immunosuppression.^{1,2,4} Induced metabolism of L-tryptophan (L-Trp) through kynurenine pathway and consequential production of kynurenine, 3-hydroxy kynurenine, kynurenic acid, excitotoxin quinolinic acid, and other metabolites are primarily responsible for local immunosuppression.^{4–6}

In nonhepatic cells, indoleamine 2,3-dioxygenase 1 (IDO1) catalyze the rate limiting step of the L-Trp catabolism through kynurenine pathway. IDO1 activity is generally low in healthy humans and has insignificant physiological effects. However, within the immune system IDO1 gets highly up-regulated in response to inflammatory signals under pathophysiological conditions (e.g., in tumor cells). Up-regulation of IDO1 is interrelated with poor prognosis in different types of cancers, including pancreatic, ovarian, colorectal, and others.^{5,6} Recent reports also suggest that neurodegenerative disorder, HIV-1 encephalitis, and other diseases are also associated with the up-regulated IDO1 activity.^{7,8} IDO1 promotes a tolerogenic state in the tumor cells and its lymph nodes by suppressing the T cells and enhancing the local regulatory T cells. IDO1

expression is strongly up-regulated by cytokines like interferon- γ . Cytotoxic T lymphocytes produce this interferon- γ , which perhaps indulges the efficacy of other immune therapy against cancer.³ A variety of preclinical studies with cancer models suggests that IDO1 assists cancer progression and metastasis.^{9,10} All these findings highlight the effectiveness of IDO1 in cancer and other diseases. Recent developments have shown that inhibition of IDO1 enzyme activity improves the efficacy of chemotherapeutic and radio therapeutic treatment of malignant tumors.^{6,7,9,10}

There are a number of reported small molecule-based IDO1 inhibitors with different structural classes, including tryptophan, imidazole, triazole, N-hydroxyamidine, iminoquinone, and others. Natural products like norharman, β -carboline, benzomalvin, and their derivatives also showed IDO1 inhibitory potencies.^{8,11–13} IDO1 inhibitors INCB024360, NLG919, and 1-methyl-L-tryptophan (L-1MT) are currently under clinical trials for the treatment of different types of cancers.^{8,14,15} Successful use of ipilimumab and nivolumab and current clinical development of the inhibitors of IDO1 enzyme inspire researchers to develop IDO1 inhibitors for cancer immunotherapy.¹⁴

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To identify a new structural class of IDO1 inhibitors that could be optimized to afford potent and selective inhibitors of IDO1, we synthesized a series of fused di- and triheterocyclic compounds. Activity studies of these compounds revealed their strong and selective IDO1 inhibitory potency under the *in vitro* conditions with no/negligible cytotoxicity.

The 4-phenyl-1,4-dihydropyridine (1), 4-phenyl-2-pyridone (2), and 4-phenyl-4H-pyran (3 and 4) derivatives were synthesized according to the reported procedures (Scheme 1).¹⁶ Condensation of the substituted aromatic aldehyde with

Scheme 1. Synthetic Scheme of 4-Phenyl-1,4-dihydropyridine, 4-Phenyl-2-pyridone, and 4-Phenyl-4H-pyran Derivatives^a

^aReagents and conditions: (a) malononitrile, 6-amino uracil, EtOH, reflux, 4 h; (b) ethyl cyanoacetate, 6-amino uracil, EtOH, reflux, 4 h; (c) 5-methyl-2,4-dihydro-3H-pyrazol-3-one, 6-amino uracil, EtOH, reflux, 4 h; (d) 5-methyl-2,4-dihydro-3H-pyrazol-3-one, EtOH, reflux, 4 h; (e) ethyl cyanoacetate, hydrazine, 5-methyl-2,4-dihydro-3H-pyrazol-3-one, 6-amino uracil, EtOH, reflux, 4 h; (f) ethyl cyanoacetate, hydrazine, EtOH, reflux, 4 h.

malononitrile and 6-amino uracil, ethyl cyanoacetate and 6-amino uracil, 5-methyl-2,4-dihydro-3H-pyrazol-3-one and 6-amino uracil, methyl-2,4-dihydro-3H-pyrazol-3-one, methyl-2,4-dihydro-3H-pyrazol-3-one, hydrazine hydrate and ethyl cyanoacetate, or hydrazine hydrate and ethyl cyanoacetate yielded these heterocyclic compounds under mild basic conditions. This single-step synthetic method is highly beneficial for structural modifications and large-scale production.

Inhibitory activity of the compounds was first explored using standard spectrophotometric method.^{8,11,17} Absorption spectra of the compounds (10 nM to 50 μ M) showed no or little interference with this enzyme activity assay. The calculated K_m and k_{cat} values of the enzyme with the L-Trp were 68.9 ± 3.5 μ M and 4.2 ± 0.2 s⁻¹, respectively. To improve the efficacy of the fused heterocyclic compounds, we investigated two general modifications of the structure of the compounds: alteration of the fused heterocyclic ring and substitution of the phenyl ring.

We first investigated the role of fused di- and triheterocyclic ring on IDO1 enzyme activity (Tables 1 and 2). The IC₅₀ values of the lead compounds 1a, 2a, 3a, and 4a suggest that

Table 1. Inhibitory Activity of the Aryl Substituted 4-Phenyl-1,4-dihydropyridine and 4-Phenyl-2-pyridone Derivatives against Purified Human IDO1 Enzyme

compd	IDO1 IC ₅₀ (nM) ^a	compd	IDO1 IC ₅₀ (nM) ^a
1a; R ₁ = R ₂ = R ₃ = H	2784 $\pm$ 72	2a; R ₁ = R ₂ = R ₃ = H	2092 $\pm$ 54
1b; R ₁ = R ₃ = H, R ₂ = F	718 $\pm$ 19	2b; R ₁ = R ₃ = H, R ₂ = F	383 $\pm$ 15
1c; R ₁ = R ₃ = H, R ₂ = Cl	473 $\pm$ 23	2c; R ₁ = R ₃ = H, R ₂ = Cl	296 $\pm$ 18
1d; R ₁ = R ₂ = H, R ₃ = F	668 $\pm$ 30	2d; R ₁ = R ₂ = H, R ₃ = F	1149 $\pm$ 41
1e; R ₁ = R ₂ = H, R ₃ = Cl	909 $\pm$ 56	2e; R ₁ = R ₂ = H, R ₃ = Cl	1365 $\pm$ 47
1f; R ₁ = R ₂ = H, R ₃ = Br	942 $\pm$ 78	2f; R ₁ = R ₂ = H, R ₃ = Br	790 $\pm$ 26
1g; R ₁ = H, R ₂ = R ₃ = F	680 $\pm$ 26	2g; R ₁ = H, R ₂ = R ₃ = F	559 $\pm$ 36
1h; R ₁ = H, R ₂ = R ₃ = Cl	577 $\pm$ 26	2h; R ₁ = H, R ₂ = R ₃ = Cl	650 $\pm$ 35
1i; R ₁ = H, R ₂ = Cl, R ₃ = F	879 $\pm$ 22	2i; R ₁ = H, R ₂ = Cl, R ₃ = F	362 $\pm$ 15

^aIC₅₀ values are the mean of three independent assays.

Table 2. Inhibitory Activity of the Aryl Substituted 4-Phenyl-4H-pyran Derivatives against Purified Human IDO1 Enzyme

compd	IDO1 IC ₅₀ (nM) ^a	compd	IDO1 IC ₅₀ (nM) ^a
3a; R ₁ = R ₂ = R ₃ = H	3275 $\pm$ 96	4e; R ₁ = R ₂ = H, R ₃ = F	479 $\pm$ 20
3b; R ₁ = R ₃ = H, R ₂ = F	1347 $\pm$ 41	4f; R ₁ = R ₂ = H, R ₃ = Cl	889 $\pm$ 32
3c; R ₁ = R ₃ = H, R ₂ = Cl	932 $\pm$ 39	4g; R ₁ = R ₂ = H, R ₃ = Br	652 $\pm$ 40
3d; R ₁ = R ₂ = H, R ₃ = F	571 $\pm$ 61	4h; R ₁ = H, R ₂ = R ₃ = F	441 $\pm$ 22
3e; R ₁ = R ₂ = H, R ₃ = Cl	862 $\pm$ 32	4i; R ₁ = H, R ₂ = R ₃ = Cl	505 $\pm$ 24
3f; R ₁ = R ₃ = H, R ₂ = Br	969 $\pm$ 55	4j; R ₁ = H, R ₂ = Cl, R ₃ = F	260 $\pm$ 11
3g; R ₁ = H, R ₂ = R ₃ = F	410 $\pm$ 24	4k; R ₁ = R ₂ = H, R ₃ = F, R ₄ = NH ₂ , R ₅ = Me	546 $\pm$ 18
3h; R ₁ = H, R ₂ = R ₃ = Cl	593 $\pm$ 35	4l; R ₁ = H, R ₂ = R ₃ = F, R ₄ = NH ₂ , R ₅ = Me	151 $\pm$ 15
3i; R ₁ = H, R ₂ = Cl, R ₃ = F	438 $\pm$ 22	4m; R ₁ = H, R ₂ = Cl, R ₃ = F, R ₄ = NH ₂ , R ₅ = Me	345 $\pm$ 21
4a; R ₁ = R ₂ = R ₃ = H	1139 $\pm$ 52	4n; R ₁ = H, R ₂ = OH, R ₃ = OMe, R ₄ = NH ₂ , R ₅ = Me	397 $\pm$ 21
4b; R ₁ = F, R ₂ = R ₃ = H	693 $\pm$ 19	4o; R ₁ = H, R ₂ = R ₃ = F, R ₄ = R ₅ = NH ₂	239 $\pm$ 27
4c; R ₁ = R ₃ = H, R ₂ = F	808 $\pm$ 22	4p; R ₁ = H, R ₂ = Cl, R ₃ = F, R ₄ = R ₅ = NH ₂	378 $\pm$ 18
4d; R ₁ = R ₃ = H, R ₂ = Cl	910 $\pm$ 16	5l ^b	91 $\pm$ 02

^aIC₅₀ values are the mean of three independent assays. ^bReported compound.¹³

not only the presence of di- and triheterocyclic ring but also substitution and electronic properties of these fused heterocyclic rings plays an important role in their inhibitory efficacy against purified IDO1 enzyme (Tables 1 and 2).

To optimize the efficacies of these fused heterocyclic compounds, we tested the substitution effect of the aryl ring on IDO1 activity. We presume that the presence of a substituted phenyl ring in the compound's core structural unit could also play an important role in their inhibitory efficacies because of their interactions with the amino acid

residues (Y126, C129, and F163) present in the active site of IDO1 enzyme.^{11,17,18} In our previous work, we successfully exploited these interactions and showed that halogen substituted aryl ring increased the inhibitory potencies of *N'*-hydroxyamides.⁸ We showed that the halogen substitution at the meta- and/or para-positions of the aryl ring of *N'*-hydroxyamides had greater impact on their IDO1 inhibitory activity. Several reports also described such benefits of halogen substituted aryl ring on the IDO1 inhibitory efficacies.^{8,14,17,19} For these fused heterocyclic compounds halogen substituents to meta- and/or para-positions of the aryl ring proved to be quite beneficial (Tables 1 and 2). Such stronger activity of the halogen substituted compounds could be due to the hydrophobic interactions, pi-stacking interaction with aromatic amino acids, or halogen bonding with the Lewis bases present within the active site.

The halogen substituted pyridopyrimidines **1b–i** and **2b–i** showed 1.5- to 7-fold stronger IDO1 inhibitory activities than the lead compounds **1a** and **2a** (Table 1). Among these tested compounds, 3-chloro substituted pyridopyrimidines **1c** (IC_{50} = 473 nM) and **2c** (IC_{50} = 296 nM) showed stronger IDO1 inhibitory activity than the other tested pyridopyrimidines. 3-Chloro-4-fluoro substituted pyridopyrimidine **2i** showed moderate inhibitory activity with IC_{50} value of 362 nM. However, no considerable preference for certain halogen substitutions at the meta- and/or para-positions were observed in pyridopyrimidines. Halogen substituted compounds with pyrazolopyranopyrimidine and dipyrzolopyran moieties showed 1.3- to 8-fold stronger IDO1 inhibitory activities than the original lead compounds **3a** and **4a**. For these compounds a substantial preference for the fluoro-substitution at the meta- and/or para-positions of the aryl ring were observed for these pyrazolopyranopyrimidines and dipyrzolopyrans (Table 2). Dipyrzolopyran **4j** showed lowest IC_{50} value of 260 nM among the tested compounds with 4-phenyl-1,4-dihydropyridine, 4-phenyl-2-pyridone, and 4-phenyl-4*H*-pyran moieties.

In addition, we also investigated how the electronic properties of the dipyrzolopyran moiety affect IDO1 inhibitory activities. In this regard, halogen substituted phenyl ring containing mono- and diamino-dipyrzolopyrans (at 3- and/or 5-position) **4k–p** were selected (Table 2). The results showed that monoamino substituted dipyrzolopyran derivative **4l** exhibited lowest IC_{50} values of 151 nM among all the investigated compounds. A comparison of the IC_{50} values of **4h**, **4l**, and **4o** clearly showed that the electronic properties of the dipyrzolopyran moiety also affect IDO1 inhibitory activities. Similar inhibitory activities were also observed among the dipyrzolopyrans **4j**, **4m**, and **4p**. Isovanillin derivative of dipyrzolopyran, **4n**, also showed strong IDO1 inhibitory activity. The aryl ring of compound **4n** contains 3-hydroxy and 4-methoxy group. However, its higher/similar IC_{50} value than the dihalogen substituted dipyrzolopyrans also suggest that halogen substitution of the aryl ring plays an important role at inhibiting IDO1 activity. The IC_{50} value of the reported potent compound 4-amino-*N*-(3-chloro-4-fluorophenyl)-*N'*-hydroxy-1,2,5-oxadiazole-3-carboxymidamide, **5l**, under the experimental conditions was 91 nM (Table 2), which is in accordance with the reported values.¹³ IDO1 inhibitor *L*-1MT, which is under clinical trial for the treatment of several types of cancer, showed IC_{50} value of 385 μ M, which is in accordance with the reported values.¹⁵

IDO1 activity in the presence of selected compounds was also analyzed by HPLC-based assay for further confirmation of

their inhibition potencies. In this assay the amount of kynurenine generated from *L*-Trp was directly measured to calculate the inhibitory activities of the compounds.⁸ The results showed that the inhibitory activities of the compounds are within 78–561 nM range (Table S1), and the differences in the IC_{50} values of the compounds are in agreement with that of measured using the spectrophotometric method. We presume that the accuracy of the methylene blue-ascorbate regeneration system to keep IDO1 in its active state (Fe^{2+}) could be the primary reason for the differences in the inhibitory activity values between the pDMAB- and HPLC-based methods.^{8,14} The HPLC-based assay also showed that monoamino substituted dipyrzolopyran derivative **4l** displayed strongest inhibitory activity (IC_{50} = 78 nM) among all the investigated compounds.

Overall, the inhibitory efficacy of the fused heterocyclic compounds is sensitive to the size and position of the halogen substituent(s) on the aryl ring, possibly due to the restricted space in “pocket A” of the IDO1 enzyme. Stronger inhibitory activities of the 3-chloro substituted pyridopyrimidines **1c** and **2c** could be due to both electronic properties of the fused diheterocyclic ring, hydrophobic interaction between the halogen substituted aryl ring, and the hydrophobic residues present within the IDO1 binding pocket. Preference for the fluoro-substitution at the meta- and/or para-positions of the aryl ring of the dipyrzolopyran derivatives (**4j** and **4l**) could be due to the pi-stacking interaction with aromatic amino acids (Y126 and F163). Electronic properties of the fused heterocyclic ring and the interactions of the fused heterocyclic ring with the heme-group and polar residues of the IDO1 enzyme plays important roles for the inhibitory potencies of the dipyrzolopyran derivatives. This could explain why dipyrzolopyran derivative **4j** or **4l** showed lower IC_{50} values than compounds **1c**, **2c**, and **3g**.

The optical properties of the heme-group are very much sensitive to the local environment and crucial in understanding the ligand binding aptitude to the IDO1 enzyme.¹⁴ Although, IDO1 enzyme inhibition studies demonstrate the inhibition capability of these fused heterocyclic compounds, but they fail to provide any direct evidence of ligand binding to the active site of the enzyme. In this regard, we recorded the absorption spectra of ferric-IDO1 and deoxy-ferrous-IDO1 in the absence and presence of the selected compounds (Figure 1A,B).¹⁴ Figure 1A showed that in the presence of compounds the Soret peak (404 nm) showed no substantial shift, indicating its trivial binding to the ferric-IDO1 enzyme. Figure 1B showed that in

Figure 1. Absorption spectra of ferric-IDO1 (A) and deoxy-ferrous-IDO1 enzyme in the absence and presence of the compounds (270 μ M) in 50 mM Tris-HCl buffer at pH 8.0. [IDO1] = 5 μ M. Ferrous-deoxy reaction environment was generated by adding $Na_2S_2O_4$ to the solution under N_2 atmosphere (B).

the absence of compound the deoxy-ferrous-IDO1 enzyme displayed Soret and Q-band at 421 and 556 nm.¹⁴ In the presence of compounds, **4j** and **4l** the Soret band (421 nm) got blue-shifted, and new Q bands appeared around 520/550 nm (Figure 1B). This indicates their probable binding to the Fe²⁺-IDO1 enzyme. The UV-vis spectra of only compounds did not show any peak in this region (Figure S1). Although additional studies are necessary to confirm the binding of these compounds to the IDO1 enzyme, but these spectral analyses undoubtedly support the binding of compounds to the ferrous-IDO1 enzyme.

To determine the therapeutic potential of these potent compounds, cellular IDO inhibitory activities were tested in MDA-MB-231 breast tumor cells. It is reported that interferon (IFN)- γ substantially promote the expression of native IDO enzyme from its mRNA in MDA-MB-231 cells.^{8,20} Calculated inhibitory activities of the compounds under these cellular environments follow similar pattern as that of against purified IDO1 enzyme. The calculated cellular EC₅₀ values of the compounds are within the range of 48–139 nM (Table 3).

Table 3. EC₅₀ Values of the Selected Compounds in MDA-MB-231 Cells

compd	MDA-MB-231 cells ^a EC ₅₀ (nM) ^b	compd	MDA-MB-231 cells ^a EC ₅₀ (nM) ^b
1c	75 $\pm$ 09	4l	48 $\pm$ 08
2c	52 $\pm$ 07	4m	81 $\pm$ 12
3g	76 $\pm$ 11	4n	113 $\pm$ 12
3i	83 $\pm$ 10	4o	73 $\pm$ 11
4e	135 $\pm$ 12	4p	139 $\pm$ 13
4h	71 $\pm$ 10	5l^c	59 $\pm$ 04
4j	52 $\pm$ 12		

^aIDO protein expression in MDA-MB-231 cells was induced by human IFN- γ (20 ng/mL). ^bEC₅₀ values are the mean of three independent assays. ^cReported compound.¹³

Control compound, **5l**, and L-1MT displayed EC₅₀ values of 59 nM and 120 μ M, respectively, under the similar experimental conditions, which are in accordance with the reported values.^{13,15} Subtle differences in the compound's inhibitory activity values between the enzymatic assay against purified IDO1 and under cellular conditions could be because of the complications in regulating the IDO1 redox activity and/or environmental effect. In general, a good correlation between these assays validates the IDO1 inhibition potencies of these fused heterocyclic compounds. We also presume that for the same reason the cell-based activity assay of the compounds shows lower EC₅₀ values than the IC₅₀ values against purified enzyme. Cytotoxicity assay of the compounds in MDA-MB-231 cells (concentrations of IC₅₀ and 2 $\times$ IC₅₀ values from the enzymatic assay) also displayed negligible level of toxicity of the compounds under the experimental conditions (Figures S2 and S3).

The inhibition studies reveal that few of these heterocyclic compounds strongly inhibit the L-Trp catabolic activity of the IDO1 enzyme. Therefore, to understand the mode of IDO1 inhibition we performed enzyme kinetics in the absence and presence of these potent compounds. The plots of [S]/V against inhibitor concentrations ([I]) showed that tested compounds **1c**, **2c**, **3i**, **4h**, **4j**, **4l**, **4m**, **4n**, and **4p** followed uncompetitive inhibition, whereas **3g** and **4o** followed competitive inhibition modes (Table S2 and Figure S4). V

and [S] represent the initial rate of the reaction and the substrate concentration, respectively.^{8,21} However, this observed uncompetitive mode of IDO1 inhibition does not demonstrate their actual mode of enzyme binding. There are several reports of uncompetitive and noncompetitive inhibitors of IDO1 enzyme. 4-Phenylimidazole is a noncompetitive inhibitor of IDO1, but its crystal structure and enzyme activity studies clearly demonstrate its binding to IDO1 active site.^{14,22} Recently reported *o*-alkylhydroxylamines are also uncompetitive inhibitors of IDO1 with activities in the nanomolar range.¹⁴ Mechanistic studies of the IDO1 induced catabolism of L-Trp revealed that the formation of ferric-superoxide intermediate is the primary requirement for this oxidation process. Hence, additional kinetic studies with respect to O₂ are needed to understand the definite mode of IDO1 enzyme inhibition by these fused heterocyclic compounds.^{8,11,14}

Our enzyme inhibition studies clearly demonstrate that the potent compounds strongly interact with the enzyme through its active site. Spectroscopic studies also supported their binding capabilities with the IDO1 enzyme. To determine their plausible mode of interactions, we performed molecular docking analyses with the IDO1 enzyme (PDB code: 4PK5).²³ The model structures propose that the pyridopyrimidines **1c** and **2c** might have two equally possible binding modes; in "pocket A" near F163 and S167 residues or "pocket B" near F226 and 7-propionate of the porphyrin ring. A similar mode of interaction for the pyrazolopyranopyrimidines **3g** and **3i** was observed. However, the molecular models predicted that dipyrzolopyrans **4j**, **4l**, and **4m** interact with IDO1 enzyme preferably through its "pocket A". The halogen substituted aryl rings of **4j** and **4l** could be involved in interaction with the amino acids like F163, F164, Y126 and others present in "pocket A" through pi-stacking and hydrophobic interactions (Figure 2). The fused triheterocyclic ring of these dipyrzolo-

Figure 2. Probable mode of interaction of the compounds **4j** (A), **4l** (B), and **4o** (C) with the active site of the IDO1 enzyme (4PK5). 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.

pyran derivatives could be involved in interaction with the heme group. This proposed interaction is in accordance with the spectroscopic based binding studies. The pyrazole ring could be also involved in interaction with S167 residue and 7-propionate group of heme, through hydrogen bonding. An additional interaction between the 3-amino pyrazole ring of compound **4l** with S167 residue was observed (Figure 2B). A similar mode of interaction was also observed for compound **4m**. Model structures also propose that the presence of additional 5-amino group of 3,5-diamino substituted dipyrzolopyran derivatives may not have any substantial role in interaction with the IDO1 through its binding site, which is in accordance with its effectiveness for IDO1 inhibitory activity

(4o). Electronic properties of the fused heterocyclic ring and halogen substitution on the aryl ring also play crucial role in binding of the compounds to the active site of IDO1 enzyme. Hence, hydrogen bonding, pi-stacking, and hydrophobic interactions play important roles in stronger binding of the compounds. The differences in inhibitory activities among the compounds under the experimental conditions suggest that their mode of interaction with the IDO1 enzyme active site could be different than the proposed one by molecular docking analysis. Molecular volume and interaction pattern of the compounds could be also critical for their binding under the experimental conditions.

TDO is the other enzyme that also catalyzes the rate-limiting step of the kynurenine pathway in the liver. TDO enzyme directly regulates the cellular level of L-Trp. Therefore, to determine the selectivity of the compounds at inhibiting the IDO1 enzyme, we measured the inhibitory activities of the selected compounds against purified TDO enzyme. Activity studies showed that these fused heterocyclic compounds have variable TDO inhibition properties. The IC₅₀ values of the selected compounds against purified TDO enzyme vary from 3 to 79 μ M (Table S3). Compound 4j and 4l showed higher inhibitory activity for IDO1 (>300-fold) over TDO enzyme under the similar experimental conditions. The results also showed that substitution of the aryl ring and electronic properties of the fused heterocyclic ring play crucial roles in their TDO inhibition. HPLC analysis also showed similar TDO inhibitory activities of these potent compounds (Table S1).

In this study, fused heterocyclic compounds were designed as IDO1 inhibitor. Subsequent modification of the electronic properties of the fused heterocyclic ring and substitution of the aryl ring directed the identification of potent inhibitors with nanomolar level IDO1 enzyme inhibitory activities under the in vitro conditions. Overall, activity studies showed that the 3,5-dimethyl-dipyrzopolopyran or 3-amino-5-methyl-dipyrzopolopyran moiety and dihalogen substituted aryl ring could considerably enhance the inhibition potency of these fused heterocyclic compounds. Spectroscopic studies suggest that the dipyrzopolopyran derivatives preferably interact with the deoxy-ferrous-IDO1 enzyme. Molecular model structure suggests that the electronic properties of dipyrzopolopyran ring and halogen substituted aryl ring assist these compounds to interact with the IDO1 through hydrogen bonding, pi-stacking, and hydrophobic interactions. Additional calculation of inhibitory constant (K_i) values from the enzyme kinetics measurements revealed that the K_i values of these potent compounds are within 35–297 nM range. Compound 4l showed the lowest K_i value of 35 nM. Reported compound 5l showed K_i value of 22 nM under the similar experimental conditions (Table S2).¹³ The strong potencies of compounds 4j and 4l also yielded very good ligand efficiencies of 0.42 and 0.43, respectively.¹⁴ Hence, dipyrzopolopyran represent a promising class of IDO1 inhibitors. The potent compounds also displayed >300-fold stronger inhibition for IDO1 enzyme inhibition in comparison with the TDO enzyme. IDO1 activity in the MDA-MB-231 cells showed that the tested compounds have minimal cytotoxicity and low-nanomolar potencies. Low cytotoxicity and inactivity for TDO enzyme support further development of dipyrzopolopyran derivatives as inhibitor of IDO1 enzyme.

In summary, to find potent inhibitors of IDO1 enzyme we synthesized a series of fused heterocyclic compounds. Several of these compounds showed strong inhibitory activity against human IDO1 enzyme. The presence of fused di- or

triheterocyclic moieties and their electronic properties could be the key factor for their strong *in vitro* inhibitory activities. Halogen substitutions in the aryl ring were effective in improving the potency of these compounds. These compounds also could be considered as good molecular probes based on their ligand efficiency values. Overall, these observations suggest that these simple fused heterocyclic compounds are potential inhibitors of IDO1 enzyme and could be of interest as drug target in cancer and other human diseases.

■ ASSOCIATED CONTENT

§ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmchemlett.6b00359.

Experimental sections, IDO1 and TDO inhibition potencies, characterization, and ¹H and ¹³C NMR spectra of the synthesized compounds (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: dmanna@iitg.ernet.in.

Author Contributions

§S.P. and A.R. contributed equally to this work.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

IDO1, indoleamine 2,3-dioxygenase 1; TDO, tryptophan 2,3-dioxygenase; L-Trp, L-tryptophan

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