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Aminoisoxazoles as potent inhibitors of tryptophan 2,3-dioxygenase 2 (TDO2)

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KEYWORDS: CANCER IMMUNOTHERAPY, IDO, TDO, HEME, ENZYME.

ABSTRACT

Tryptophan 2,3-dioxygenase 2 (TDO2) catalyzes the conversion of tryptophan to the immunosuppressive metabolite kynurenine. TDO2 over-expression has been observed in a number of cancers; therefore, TDO inhibition may be a useful therapeutic intervention for cancers. We identified an aminoisoxazole series as potent TDO2 inhibitors from a high-throughput screen (HTS). An extensive medicinal chemistry effort revealed that both the amino group and the isoxazole moiety are important for TDO2 inhibitory activity. Computational modeling yielded a binding hypothesis and provided insight into the observed structure-activity relationships. The optimized compound **21** is a potent TDO2 inhibitor with modest selectivity over indolamine 2,3-dioxygenase 1 (IDO1) and with improved human whole blood stability.

INTRODUCTION

One of the critical functions of the immune system is to detect and eliminate malignant cells as they arise and develop antigenic mutations. For tumor cells to survive, the host immune system must be rendered functionally tolerant to otherwise immunogenic tumor-associated antigens. To accomplish this, tumors have been shown to enlist an array of endogenous host regulatory pathways.[1] One of these pathways is mediated by catabolism of the essential amino acid tryptophan (Trp) into kynurenine (Kyn), a process driven by the rate-limiting enzymes indoleamine-2,3-dioxygenase 1 (IDO1) and Trp-2,3-dioxygenase 2 (TDO2).[2] These enzymes catalyze the oxidation of L-tryptophan (Trp) to N-formyl kynurenine (NFK), which rapidly converts to kynurenine (Kyn). Over-expression of these enzymes has been observed in several cancers and is thought to mitigate the immune response towards these tumors.[3, 4]

The enzymatic functions of IDO1 and TDO2 lead to multiple immune-related responses. IDO1-mediated Trp depletion activates GCN2 kinase,[5] which in turn phosphorylates and inactivates the transcription factor eIF2α leading to the blockade of protein translation and inhibition of cell proliferation. Kyn accumulation on the other hand activates the aryl hydrocarbon receptor (AhR),[6] a transcription factor implicated in cancer progression, invasion and immune suppression.[2] The overall result of IDO1 activity is induction of the regulatory T cell (Treg) compartment of the immune system, with concomitant inhibition of the effector T cell (Teff) compartment. IDO1-deficiency (gene ablation or small molecule inhibition) has been shown to boost Teff and decrease Treg function in several autoimmunity and *in vivo* tumor models.[7, 8, 9]

The physiological function of TDO2 includes the regulation of systemic Trp levels as well as liver Kyn levels. *In vitro* and *in vivo* studies suggest that TDO2 plays a role in promoting tumor cell survival and motility, and that TDO2-expressing tumors have a lower degree of T cell infiltrate that allows them to escape immune rejection.[6, 10] Genetic knockout or treatment of mice with a tool enzymatic inhibitor of TDO2 activity resulted in increased sensitivity of mice to endotoxin-induced shock, consistent with a role for TDO2 in suppressing inflammation and maintaining immune homeostasis.[11] In tumors, TDO2 activity is highly expressed in liver cancer and glioblastoma and upregulated in several other cancers.[6, 12] Thus IDO1 and TDO2 inhibitors may represent a useful therapeutic intervention that restores the immune response against tumors, especially when combined with immune checkpoint inhibitors. Indeed, nonclinical data demonstrates that IDO1 and anti-PD-L1 dual inhibition shows markedly greater efficacy in activating the immune system and inhibiting tumor growth than either treatment alone, with a response associated with more pronounced activation of intratumoral CD8+ T cells, including proliferation and cytokine production.[13, 14] Preliminary data of a phase I/II clinical trial in melanoma where the IDO1 inhibitor

epacadostat is being tested in combination with anti-PD1 has shown promising results in terms of response rates and depth of responses, compared to anti-PD-1 data.[15]

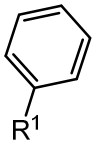
While several classes of IDO1 inhibitors have been disclosed in the literature,[16,17,18,19,20], at the onset of our program, TDO2 inhibitors had been explored to a far lesser extent.[21] Several patent applications describing either TDO-selective or IDO/TDO dual inhibitors have appeared since.[22,23,24,25,26] TDO2 inhibition alone or in combination with IDO1 inhibition may allow fuller suppression of the kynurenine pathway across a range of tumor types. Here we report our efforts in identifying and optimizing aminoisoxazole-based TDO2 inhibitors.

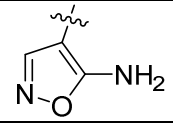
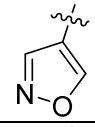
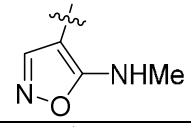
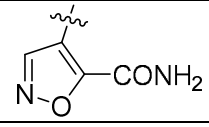
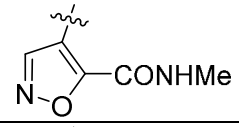
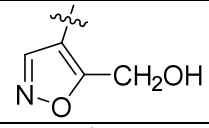
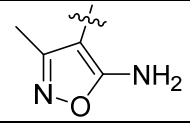
RESULTS

A high-throughput screen of the Genentech compound library identified a number of related aminoisoxazoles as inhibitors of TDO2. Inhibitory potency was measured in both biochemical and cellular assays of IDO1 and TDO2. In the biochemical assays, inhibitors were incubated with recombinant enzymes in the presence of the substrate tryptophan. The production of the N-formylkynurenine (NFK) was detected either in a fluorescence- or mass spec-based assay and inhibitory potency was expressed as IC_{50} values. In the cellular assays, inhibitors were incubated with either SW48 cells (in which endogenous TDO2 is highly expressed) or A172 cells (in which IDO1 is highly expressed upon induction by IFNγ). The production of NFK released from cells into the media was detected by a fluorescence-based assay and the inhibitory potency was expressed as EC_{50} values. The cytotoxicity of compounds was also measured for the same treated cells with a luminescence-based assay detecting cellular ATP levels. Since TDO2 biochemical potency was poorly predictive of cellular potency for the aminoisoxazole compounds (see Figure S1 in supplemental material), our medicinal chemistry designs were consequently guided by cellular potency. As a counter screen to identify inhibition as a result of cytotoxicity, we assessed cell viability of SW48 and A172 cells by measuring intracellular ATP concentration. We found that the aminoisoxazoles generally do not affect intracellular ATP concentrations up to 25 μM inhibitor concentration in a cell viability assay, indicating that the cellular potency was not caused by cytotoxicity of the inhibitors.

Aminoisoxazole **1** was the most potent hit found hit (cellular EC_{50} of 85 nM, Table 1). We first investigated the importance of the amino group on the isoxazole moiety. Removal of the amino group (**2**) resulted in complete loss of potency. Addition of a methyl group on the nitrogen atom (**3**) resulted in a modest ~8-fold reduction of potency. Replacement with a primary amide (**4**), secondary amide (**5**), hydroxymethyl group (**6**), or addition of a methyl group to the isoxazole 3-position (**7**) all resulted in significant reduction of TDO2 inhibitory potency.

Table 1 TDO2 cellular and biochemical potencies of the aminoisoxazole modifications



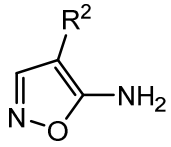
Compound	R^1	TDO2 EC_{50} (μM)[a]	TDO2 IC_{50} (μM)[b]
1		0.085	0.14
2		>25	>25
3		0.67	0.19
4		>25	>25
5		>25	>25
6		18	4.4
7		9.7	25

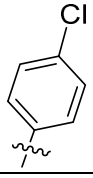
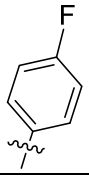
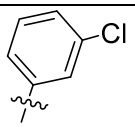
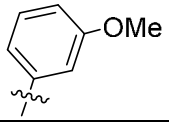
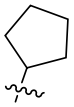
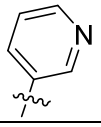
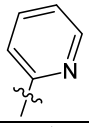
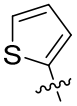
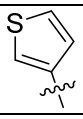
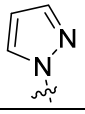
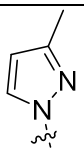
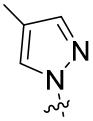
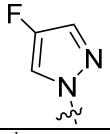
[a]Cellular potency. [b]Biochemical potency. IC50 and EC50 values are the averages of at least 2 independent experiments.

We next investigated substitution and replacement of the phenyl moiety of compound **1**. Introduction of *p*-Cl (**8**), *p*-F (**9**), *m*-Cl (**10**), or *m*-methoxy (**11**) on the phenyl ring reduced TDO2 cell potency (Table 2). Replacing the phenyl group with a cyclopentyl (**12**) resulted in ~4-fold reduction of potency while a cyclohexyl (**13**) reduced the potency by 18-fold. Since more hydrophilic compounds tend to have better metabolic stability, we were interested in introducing groups that are more polar than the phenyl group. Pyridyl analogs (**14** and **15**) resulted in more than 10-fold reduction of potency. Between the two thiophenyl regio-isomers, cellular potency of 3-thiophenyl **17** was much better than 2-thiophenyl **16**. The unsubstituted pyrazole (**18**) was ~2.6-fold less potent than the corresponding phenyl analog **1**, while addition of a methyl group at either the C3- or C4- of the pyrazole (**19** and **20**) resulted in further erosion of TDO2 potency. However, introduction of a fluorine substituent at C4 of the pyrazole led

to the most potent TDO2 inhibitor **21** within the aminoisoxazole series, with a cellular EC_{50} of 77 nM.

Table 2 TDO2 cellular and biochemical potencies of phenyl ring substitution or replacements

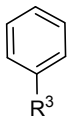


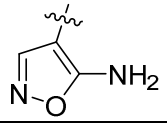
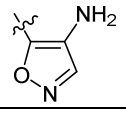
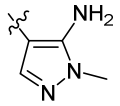
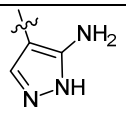
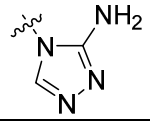
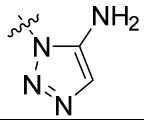
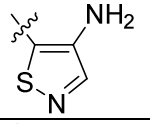
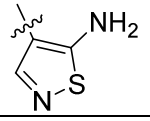
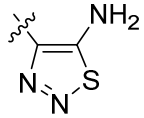
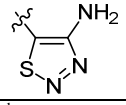
Compound	R^2	TDO2 EC_{50} (μM)[a]	TDO2 IC_{50} (μM)[b]
8		0.95	0.83
9		0.21	0.58
10		0.25	0.50
11		1.5	3.1
12		0.36	0.64
13		1.5	7.8
14		1.4	2.3
15		0.82	1.7
16		0.81	0.029
17		0.099	0.028
18		0.23	0.29
19		0.81	1.6
20		0.34	1.6
21		0.077	0.34

[a]Cellular potency. [b]Biochemical potency. IC50 and EC50 values are the averages of at least 2 independent experiments.

Because the isoxazole core was found to exhibit poor stability in whole blood (*vide infra)*, we investigated replacement of the 5-aminoisoxazole moiety with alternate heterocycles. Given that the amino group is required in the 5-aminoisoxazole for TDO2 potency (Table 1), the amino group was retained in all the designed heterocyclic targets. However, all azoles tested, including 4-aminoisoxazole (**22**), 3-aminopyrazoles (**23** and **24**), 3-amino-1,2,4-triazole (**25**), 4-amino-1,2,3-triazole (**26**), 4-aminoisothiazole (**27**), 5-aminoisothiazole (**28**), 5-amino-1,2,3-thiadiazole (**29**) and 4-amino-1,2,3-thiadiazole (**30**) were inactive against TDO2 (Table 3). We concluded that the 5-aminoisoxazole is a structurally unique core that is required for TDO2 inhibition.

Table 3. TDO2 cellular and biochemical potencies of isoxazole replacement



Compound	R^3	TDO2 EC_{50} (μM) [a]	TDO2 IC_{50} (μM) [b]
1		0.085	0.14
22		>25	>25
23		>25	>25
24		>25	>25
25		>25	>25
26		>25	>25
27		>25	>25
28		>25	18
29		>25	>25
30		>25	0.27

[a]Cellular potency. [b]Biochemical potency. IC50 and EC50 values are the averages of at least 2 independent experiments.

To better understand the structure-activity relationships and develop a binding mode hypothesis, we constructed a model of **21** bound to human TDO2 (Figure 1) using the X-ray crystal structure, PDB 5TI9.[27] Our model predicts a binding mode with interactions similar to those of tryptophan. The primary amine, isoxazole ring nitrogen and fluoropyrazole moiety in **21** correspond with the ammonium, carboxylate, and indole ring of tryptophan, respectively. In addition, the internal strain energy in this pose was found to be at the global energy minimum using quantum mechanical calculations (supplemental material Figure S2). The strong interaction between the primary amine and the heme propionic acid in the model of **21** could explain the requirement for a primary or secondary amine as observed in Table 1. Small, hydrophobic replacements of the 4-fluoropyrazole in **21** would fit in the pocket (Table 2) while larger substituents such as the methoxy group in **11** would extend further than the tryptophan indole to experience a steric clash, thus explaining the increase in both IC_{50} and EC_{50} of **11**. Finally, the model predicts important interactions between the isoxazole heteroatoms and Arg144 which could explain the general loss of potency when either of these atoms were replaced with hydrogen-bond donors or were methylated as in **22-24** and **26-27.** The predicted interactions with Arg144 may also explain the measurable biochemical activities in thiazole **28** or thiadiazole **30**. Triazole **25** maintained the two hydrogen bond acceptors of the isoxazole but the nitrogen-linked ring was predicted to adopt an alternate low-energy conformation (Figure S2) compared to **1** or **21**. The complete loss of cell potency of all isoxazole substitutions cannot be fully explained by the model. However, this model is consistent with our observation that the aminoisoxazole did not cause perturbation of the maximal UV absorption wavelength when incubated with IDO1 or TDO2 (data not shown), suggesting that it does not bind the heme iron directly. In addition, TDO2 biochemical IC_{50} values increased when a higher tryptophan concentration was used, suggesting that isoxazoles are competitive with the tryptophan substrate. For example, biochemical IC_{50} of compound **8** shifted from 0.83 μM to 4.21 μM when tryptophan concentration was increased from 0.2 mM to 1 mM. IC_{50} of compound **10** shifted from 0.50 μM to 2.94 μM when tryptophan concentration was increased from 0.2 mM to 1 mM.

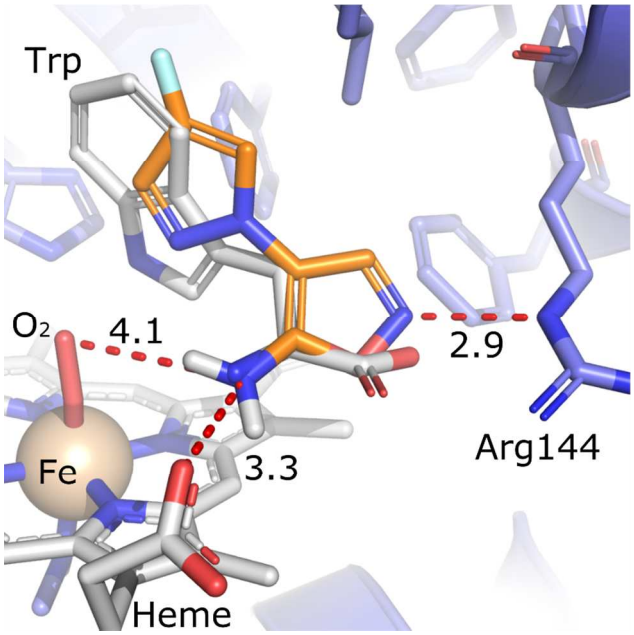


Figure 1. Binding mode hypothesis for compound 21. X-ray structure (PDB: 5TI9) of human TDO2 (purple) is shown with heme (gray), Tryptophan substrate (gray), and O_2 (red). A putative bound conformation for compound **21** (orange) was then modeled (see methods in the supplement for details). Distances are in angstroms.

Selected compounds were also tested in an IDO1 cell-based assay. As shown in Table 4, the compounds showed modest TDO2 selectivity over IDO1, ranging from 6- to 14-fold.

Table 4. TDO2 selectivity over IDO1

Compound	TDO2 selectivity[a] (fold)
1	13x
3	12x
17	14x
21	6x

[a]Calculated as IDO1 EC_{50}/TDO2 EC_{50}.

As TDO2 is a heme-containing enzyme, we tested the compounds in cytochrome P450 (CYP) inhibition assays and found that the 4-aminoisoxazole analogs did not inhibit common drug-metabolizing CYP enzymes. For instance, the most potent compounds **1**, **17**, **18** and **21** all had IC_{50}'s greater than 10 uM for all the CYP isoforms that were tested (CYP3A4, CYP1A2, CYP2D6, CYP2C9 and CYP2C19).

We also investigated the stability of select compounds in whole blood to predict the *in vivo* stability (Table 5). The original HTS hit **1**, the N-methylated analog **3** and thiophene **17** demonstrated poor stability in rat, dog and human whole blood. The 3-methylisoxazole analog **7** showed improved whole blood stability but with significantly reduced TDO2 potency. However, pyrazole analog **21** demonstrated improved human whole blood stability while maintaining TDO2 potency. The cause of the observed whole blood instability is not well understood at this time.[28]

Table 5. Whole blood stability[a]

Compound	% remaining rat	% remaining dog	% remaining human
1	0	0	0
3	0.1	0.1	0.3
17[b]	3	3	NT
7	50	65	84
21	NT	NT	91

[a]Compounds (2 uL, 0.5 mM) were incubated in 100 µL of fresh blood for 2 hours unless otherwise stated. Parent remaining is analyzed by LC-MS/MS and reported as a percentage. NT: not tested. [b]Incubated for 30 minutes.

CONCLUSION

In summary, high-throughput screening identified a series of 5-aminoisoxazole compounds as potent TDO2 inhibitors. Medicinal chemistry investigations revealed that both the amino group and the isoxazole moiety were important for TDO2 inhibitory activity, and a hypothetical binding mode was developed through molecular modeling, providing insight into the observed SAR. Optimized compound **21** is a potent TDO2 inhibitor with modest selectivity over IDO1, no observed CYP inhibition, and improved human whole blood stability over the original HTS hit. However, the mechanism of whole blood instability for this series, and relationship with structure, remained unclear. As a result, this series was deprioritized for further optimization in light of alternative more promising chemical matters.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website and includes experimental procedures, cell versus biochemical potencies and quantum mechanical torsional energy analysis

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

IDO, indoleamine 2,3-dioxegenase TDO, tryptophan 2,3-dioxegenase; NFK, N-formyl kynurenine, Kyn, kynurenine, AhR, aryl hydrocarbon receptor; Treg, regulatory T cell; Teff, T cell; P450, cytochrome P450

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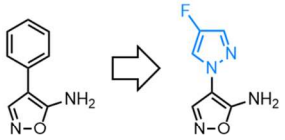
(TOC Image)



Cell EC_{50}	85nM	77nM
IDO1 Select.	13x	6x
Whole Blood (% remain.)	0 %	91 %