# Rational Design of Original Fused-Cycle Selective Inhibitors of Tryptophan 2,3-Dioxygenase

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**ABSTRACT:** Tryptophan 2,3-dioxygenase (TDO2) is a heme-containing enzyme constitutively expressed at high concentrations in the liver and responsible for L-tryptophan (L-Trp) homeostasis. Expression of TDO2 in cancer cells results in the inhibition of immune-mediated tumor rejection due to an enhancement of L-Trp catabolism *via* the kynurenine pathway. In the study herein, we disclose a new 6-(1H-indol-3-yl)-benzotriazole scaffold of TDO2 inhibitors developed through rational design, starting from existing inhibitors. Rigidification of the initial scaffold led to the synthesis of stable compounds displaying a nanomolar cellular potency and a better understanding of the structural modulations that can be accommodated inside the active site of *h*TDO2.

# INTRODUCTION

Multiple immune resistance mechanisms are often involved within a tumor microenvironment, hampering the immune response and leading to the failure of currently available treatments.<sup>1,2</sup> As we progress toward an individualized treatment approach, there is a need for new tools to overcome these hurdles. Using small-molecule inhibitors (SMIs) to target tolerogenic pathways is an attractive strategy for cancer immunotherapy. The advantages of SMIs reside in their ability to reach a wide array of intracellular targets, the possibility to fine-tune their absorption, distribution, metabolism, and excretion (ADME) properties, and their superior costeffectiveness compared to biologics.<sup>3,4</sup> Within this context, small-molecular scaffolds are a rapidly expanding area of research, as exemplified by the increasing number of patent applications and compounds currently under evaluation at preclinical and clinical stages both as stand-alone or combination therapies.<sup>3–</sup>

Enzymes of the tryptophan-kynurenine pathway (KP), such as indoleamine-2,3-dioxygenase 1 (IDO1), tryptophan 2,3dioxygenase (TDO2), or kynurenine 3-monooxygenase (KMO), are attractive drug targets for cancer immunotherapy.<sup>6,7</sup> Catabolism of L-Trp promotes an immunosuppressive environment both through the depletion of this essential amino acid and the accumulation of kynurenine (KYN) metabolites.<sup>8</sup> First, a decrease in L-Trp levels results in the blockage of CD8+ and CD4+ T-cells, in the G1 cell cycle phase, due to their susceptibility to amino acid shortage.<sup>9</sup> Second, it leads to the activation of the general control nonderepressible 2 (GCN2) "stress" sensing kinase, further hindering CD8+ T-cell proliferation.<sup>10</sup> Finally, immunosuppression from L-Trp deprivation might also be linked to the inhibition of the mammalian target of the rapamycin (mTOR) pathway.<sup>11</sup>

On the other hand, along with its catabolites, KYN favors regulatory T cells (Tregs) through several mechanisms. One of the currently proposed mechanisms resides in the interaction between KYN and the aryl hydrocarbon receptor (AHR), leading to the polarization of CD4+ T-cells into the Treg phenotype.  $^{12-14}$ 

Overall, excessive KP activation promotes carcinogenesis and tumor cell growth by reducing T-cell proliferation, suppressing natural killer cells (NK), promoting Tregs differentiation, and activating, recruiting, and expanding myeloid-derived suppressor cells (MDSCs).<sup>15,16</sup>

This implication of the KP in tumor immune escape has led to considerable efforts from the medicinal chemist community to develop SMIs to interfere with this pathway. Most notably, the development of SMIs for IDO1 has received tremendous

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hTDO2 (μM)	IC₅₀(enz) <sup>a</sup>	K <sub>i</sub> 0.88 <sup>d</sup>	[6.1-8.7]	[5.6 – 7.1]	[4.9 – 6.6]	[12.8 – 16.9]	[21.2 – 32.6]	[12.7 – 22.9]	[2.0-3.4]
	IC₅₀(cell) <sup>a</sup>	0.28 <sup>d</sup>	[1.74 – 6.62]	[0.07 – 0.12]	[0.10 - 0.14]	[0.27 – 0.40]	[0.49 – 0.66]	[0.83 – 1.16]	[0.70 – 1.02]
hIDO1 (μM)	IC₅₀(enz)	NI <sup>e</sup>	NI						
	IC₅₀(cell)								
LD50	<sup>b</sup> (μM)	> 80 <sup>d</sup>	> 100	> 20	> 30	> 100	> 100	> 30	> 100
cLo	<b>gD</b> <sub>7.4</sub> <sup><i>c</i></sup>	3.34	0.15	3.49	3.49	3.17	3.17	2.60	3.01
L	LE	2.72	4.99	1.76	1.75	1.67	1.46	2.18	2.56
LE		0.46	0.41	0.38	0.38	0.35	0.33	0.33	0.42

Table 1. 680C91, LM10, and their Fused-Cycle Analogues (1-6) with their Inhibition Data

<sup>*a*</sup>95% confidence intervals. Reported IC<sub>50</sub> values are calculated from measurements performed in triplicates, in at least two separate experiments. <sup>*b*</sup>Toxicity was determined using an MTS assay on P815B cells. <sup>*c*</sup>log  $D_{7,4}$  values were calculated using Chemicalize. <sup>*d*</sup>Previously obtained data from Pilotte et al.<sup>24</sup> <sup>*e*</sup>NI: no inhibition at 100  $\mu$ M.

attention since the discovery of its implications in cancer immune escape.<sup>17,18</sup> Although the outcomes of IDO1 inhibitors in clinical trials did not meet expectations for several possible reasons discussed in a recently published review, the KP remains a reservoir of attractive targets and an active research area.<sup>19,20</sup>

Much like IDO1, TDO2 independently catalyzes the first and rate-limiting step of the KP: the oxidative cleavage of the L-Trp pyrrole moiety to form *N*-formylkynurenine. TDO2 plays a central role in the regulation of the systemic L-Trp concentration.<sup>21</sup> Despite sharing the same enzymatic reaction, TDO2 only shares around 10% sequence identity with IDO1 and is much more specific for L-Trp.<sup>22,23</sup> Numerous human tumors, including glioblastoma, melanoma, and liver, bladder, pancreatic, and colon carcinomas, express TDO2 at various levels to exploit the KP as an immune escape mechanism.<sup>24,25</sup> TDO2 inhibitors would be particularly relevant in hepatocarcinoma, 100% of which express this enzyme.<sup>24,26</sup>

This interest in TDO2 inhibition led to the development of several families of TDO2 inhibitors, as exemplified by some patents and few articles published to date.<sup>27-30</sup> We previously established initial structure-activity relationships (SARs) for a series of TDO2-selective and competitive inhibitors characterized by a 3-(2-(pyridyl)ethenyl)indole scaffold that demonstrated several prerequisites for TDO2 inhibition and potential for optimization.<sup>31</sup> A thorough exploration of SARs around the initial **680C91** inhibitor (Table 1) drew the first prerequisite for TDO2 inhibition by catalytic-site inhibitors. To summarize, all investigated replacements of the indole moiety led to inactive compounds, even when changes were minor (5-azaindole or indazole). Moreover, the indole nitrogen should remain free, and only substitution in C5 or C6 with small lipophilic substituents such as fluorine led to increased potency. As suggested by molecular docking, the 3-pyridyl group of 680C91 is found in the vicinity of Arg-144 guanidinium. Accordingly, the replacement of this 3-pyridyl with H-bond acceptors or negatively charged groups provided more soluble compounds that could be further stabilized in the TDO2 binding cleft through ionic interactions.<sup>31</sup> Replacement of the *trans*-vinyl linker with a longer, shorter, or more flexible ethyl spacer resulted in inactive or poorly active derivatives. Thus, it appeared that TDO2 was particularly susceptible to small modifications of the inhibitor. Recent crystallographic studies revealed that the active site of hTDO2 was, in fact, more rigid than that of hIDO1, which has a broader substrate specificity.<sup>32</sup>

Hence, our previous research eventually led to the identification of LM10 (Figure 1), characterized by a *trans*-



**Figure 1.** Molecular modeling of **LM10** inside the *h*TDO2 active site (PDB code 5TI9)<sup>39</sup> and optimization strategy considered in this work.

vinyl tetrazole side chain in the 3-position of a 6-fluoroindole scaffold and a low micromolar ( $\mu$ M) potency. The administration of **LM10** restored the immune response and promoted tumor rejection in a mouse model.<sup>24</sup> **LM10** was, to our knowledge, the very first soluble and bioavailable TDO2 inhibitor demonstrating preclinical antitumor efficacy and thus the proof-of-concept of TDO2 inhibition for anticancer immunotherapy.<sup>24</sup> Lately, inhibition of TDO2 was also demonstrated to be beneficial for the efficacy of immune checkpoint inhibitors such as *anti*-PD1 and *anti*-CTLA4 monoclonal antibodies.<sup>25</sup> Interestingly, the benefit of a TDO2



Figure 2. Molecular modeling of 6 inside the hTDO2 active site (PDB code 5TI9)<sup>39</sup> and envisioned modulations around this scaffold.

blockade persisted even in the absence of its tumoral expression, probably due to the increased availability of circulating L-Trp levels.<sup>25</sup>

Our previous work provided useful data for TDO2 inhibitor development, exemplified by numerous publications and patents reporting TDO2 inhibitors based on the 6-F-indole pharmacophore.<sup>27</sup> However, the **LM10** series suffered from several drawbacks, which prevented its further therapeutic application. First, the TDO2 inhibitory potency in this series could not be much improved and could not reach a cellular efficacy below the  $\mu$ M threshold. Furthermore, we could not find a replacement for the electrophilic vinyl linker prone to chemical instability (trans–cis isomerization), side reactions (Michael acceptor), and metabolic liabilities. In the present work, to further expand SARs data for TDO2 and provide a better molecular framework for TDO2 inhibitors, we designed, synthesized, and evaluated a series of original, highly stable, and selective TDO2 inhibitors.

### RESULTS AND DISCUSSION

Design of TDO2 Inhibitors. We first reasoned that the rigidification of the lateral trans-vinyl in the 680C91 and LM10 scaffolds could improve the TDO2 inhibitory potency by conformational restriction and provide a molecular template devoid of chemical liabilities (Figure 1). Moreover, several fused-cycle scaffolds were previously reported in a patent regarding TDO2 inhibitors, suggesting that such rigidification could lead to potent TDO2 inhibitors.33 To investigate the impact of this replacement, we synthesized several fused-cycle analogues of 680C91 and LM10 (1-6, Table 1). As indicated by our previous results, fluorine substitution in the 6-position of indole usually leads to a slight increase in the TDO2 inhibitory potency.<sup>31</sup> However, we first decided to avoid 6-fluorine derivatives as their synthesis results, in our experience, in lower yields compared to their nonfluorinated counterparts. Evaluation of these compounds in enzyme and cell-based assays on IDO1 and TDO2 demonstrated their ability to selectively inhibit TDO2. Interestingly, inhibition data on isolated enzyme assay are often lacking for reported TDO2 inhibitors. Indeed, several reported series of TDO2 inhibitors were characterized only by cell-based inhibition assays, which limits the SAR understanding and hence the future development of inhibitors.<sup>27,28</sup> We used the most commonly described assay for IDO1/TDO2 activity which follows the formation of the enzymatic product N'-formylkynurenine after hydrolysis with trichloroacetic acid and reaction with p-dimethylaminobenzaldehyde.<sup>34,35</sup> Regarding cell-based evaluation, we used a

previously described assay using the hTDO2-expressing P815B murine mastocytoma cell line.<sup>24</sup>

Compounds 1 to 6 all exhibited low- $\mu$ M range enzymatic IC<sub>50</sub>s and mid-nanomolar (nM) to low- $\mu$ M activities in cells. Interestingly, these compounds also presented very low cellular toxicity, as assessed by our MTS assay (Table 1).

To rank and compare these initial hits, we normalized their respective affinities using ligand efficiency [LE:  $1.4(-\log IC_{50})/(\text{heavy atom count})$ ] and lipophilic ligand efficiency (LLE:  $\text{pIC}_{50} - c \log D_{7.4})$  metrics.<sup>36,37</sup> These values allow us to consider both activity and lipophilicity,<sup>2</sup> an equally important attribute in drug design affecting ligand association and ADME properties.<sup>38</sup>

Isoquinoline analogues 1 and 2 were the most active compounds in cells, whereas quinazolines (3, 4) showed a loss in their potency and LE/LLE values. Despite being less potent in cells than 1 and 2, hit compound 6, bearing a triazole moiety, had the lowest IC<sub>50</sub>(enz) on *h*TDO2 and overall more favorable metrics due to its higher hydrophilicity and heteroatom count. In addition to the absence of cell toxicity even at high concentrations (>100  $\mu$ M), incubation of cells with 6 did not affect cell cycle distribution, which was determined by flow cytometry with propidium iodide DNA staining (PI) (Figure S1).

Evaluation of hTDO2 inhibition by 6 demonstrated an  $IC_{50}(CI_{95\%})$  of 2.7  $\mu$ M in the enzymatic assay ( $IC_{50}(enz)$ ) and 0.8  $\mu$ M in cell-based settings (IC<sub>50</sub>(cell)), while LM10 had an  $IC_{50}(enz)$  of 7.3  $\mu$ M and an  $IC_{50}(cell)$  of 2.8  $\mu$ M. Consequently, we decided to use 6 as a starting point for designing various Nsubstituted original indole 3-benzotriazoles (Figure 2). However, compound 6 displayed worsened parameters (LLE = 2.56,  $c \log D_{7,4} = 3.01$ ) compared to LM10 (LLE = 4.99,  $c \log D_{7,4} =$ 0.15), due to the loss of the tetrazole function, ionized at a physiological pH of 7.4. Thus, our optimization included benzotriazole substitutions of various hydrophobicity ranges aimed at probing TDO2 inhibition while considering their potency and physicochemical characteristics. Then, we further modulated the benzotriazole ring by (i) replacement with a triazolo-pyridine scaffold and (ii) substitution on the 6membered ring of benzotriazole.

**SARs of Benzotriazole Analogues.** We next synthesized a chemical library of benzotriazole analogues and evaluated the synthesized compounds for their ability to inhibit TDO2 both *in vitro*, using a purified truncated form of TDO2, and *in cellulo* using TDO2-expressing P815B cells.<sup>23</sup> To remain under the initial velocity conditions, the assay was optimized so that the cells consume less than 25% of the available L-Trp. The same

Table 2. Inhibitory Affinities (IC<sub>50</sub>) of Compounds Against hTDO2 in the Spectrophotometric Assay and P815B Cells

R<sub>1</sub>

				x	N X				X - N - N - N - N - N - N - N - N - N -			
				K J		R <sub>1</sub>	K_					
			$\land$	٦´	R <sub>2</sub>	R <sub>2</sub>		R <sub>2</sub>				
		Y'	~	H	Y V N		~	H				
			N	3	N <sub>1</sub>		N	2				
N°	Ν.	v	x	Ra	R,	IC50 <sup><i>a</i></sup>	<b>ΙС</b> 50 <sup><i>a</i></sup> (μM)		LLE	IF		
	Π¥χ		~	N2		Enz.	Cell	LUGD7.4				
7	N <sub>2</sub>					4.0	0.17	3.23	2.17	0.39		
8	N <sub>3</sub>					3.87	0.22	3.17	2.24	0.39		
9 10				NL		0.02 2.9E	0.38	3.17	2.05	0.38		
11	N <sub>2</sub>					5.05	0.42	2.54	2.96	0.37		
12	N1			NH <sub>2</sub>	∗ CH₃	3.93	0.97	2.34	3.07	0.37		
13	N2		N			13.06	0.48	2.73	2.15	0.33		
14	N <sub>3</sub>		N			7.83	0.40	2.71	2.40	0.35		
15	N1		Ν			9.08	0.71	2.66	2.38	0.35		
16	N1			$NO_2$		1.32 <sup>c</sup>	0.54	2.52	3.36	0.37		
8a	N3	F				1.1	0.02	3.31	2.65	0.41		
17	N3				*~	15.0	0.44	1 13	0.69	0.29		
18	N1					1.9	0.19	4.15	1.59	0.34		
19	N <sub>3</sub>				. ^	13.5	0.98	2.05	0.92	0.30		
20	N1				*	3.9	0.10	3.95	1.46	0.34		
21	N <sub>3</sub>					39.7	1.62		2.56	0.28		
22	<b>N</b> 1				* CONH <sub>2</sub>	36.1	0.68	1.84	2.60	0.28		
23	N <sub>3</sub>				~	173.8	NI <sup>d</sup>		4.76	0.22		
24	$N_1$				* `COOH	104.5	NI	- 1.00	4.98	0.25		
25	N₃				Ţ	36.9	1.93	1 70	2.73	0.26		
26	$N_1$				*~~_N~	24.6	1.64	1.70	2.91	0.27		
27	N <sub>3</sub>				-0	44.1	1.86		1.16	0.25		
28	N1				*	21.9	0.41	3.19	1.44	0.26		
29	N <sub>3</sub>				0~\	36.9	2.53		0.81	0.25		
30	N1				*	21.1	0.50	3.59	1.08	0.27		
31	Na				$\sim$	118.6	3.73		1.20	0.21		
32	N <sub>1</sub>					52.6	1 60	2.84	1 46	0.23		
22	N				*/ \	17 72	0.02		4.25	0.25		
	IN3					17.75	0.95	0.50	4.25	0.20		
34	N1					25.51	1.15		4.09	0.25		
35	N <sub>3</sub>				*	26.10	0.62	- 0.28	4.86	0.27		
36	N1					45.65	1.78	0.20	4.62	0.26		
37	N <sub>3</sub>					24.9	0.74	0.06	3.64	0.24		
38	<b>N</b> 1					45.0	1.43	0.90	3.39	0.23		
37a	N₃ F				*	5.2	0.23	0.97	4.32	0.28		
39	N <sub>3</sub>				*ОН	17.0	0.75	4.65	2.64	0.27		
40	N1				OH	25.2	2.27	1.85	2.35	0.25		

<sup>*a*</sup>Reported IC<sub>50</sub> values are calculated from measurements performed in triplicates, in at least two separate experiments. 95% confidence intervals of the values can be found in the Supporting Information. <sup>*b*</sup>log  $D_{7,4}$  values were calculated using Chemicalize. <sup>*c*</sup>Residual activity of ~30%. <sup>*d*</sup>NI: no inhibition. Unless indicated otherwise, Y and R<sub>2</sub> = H, X = CH.

evaluation was performed on IDO1 to assess the selectivity toward TDO2 and demonstrated that none of these compounds could inhibit IDO1 (data not shown).

The active site of TDO2 is highly hydrophobic and relatively small, suggesting that an increase in potency could be achieved by adding lipophilic substituents to the initial scaffold of **6** [IC<sub>50</sub>(enz): 2.7  $\mu$ M/IC<sub>50</sub>(cell): 0.8  $\mu$ M]. To that end, we

examined if the N-alkylation of the benzotriazole moiety would result in more potent compounds. Interestingly, The N-methyl analogues (7, 8, and 9) displayed a significant gain in  $IC_{50}$  (cell) (up to 5-fold for 7) compared to their parent compound 6, while maintaining a similar  $IC_{50}$  (enz) (Table 2). These results can be explained by the increase in the log  $D_{7,4}$  value resulting from Nmethylation. For SAR purposes, we next took an interest in



**Figure 3.** Epitope mapping for regioisomers **32** [IC<sub>50</sub> 52.6  $\mu$ M (enz), 1.6  $\mu$ M (cell)] and **31** [IC<sub>50</sub> 118.6  $\mu$ M (enz), 3.7  $\mu$ M (cell)]. One-dimensional (1D) NMR <sup>1</sup>H proton spectra (blue) overlaid with STD spectra (orange). Corresponding compounds are represented above each set of spectra. Blue circles represent the protons at the designated position(s), with percentages representing STD intensity of the respective proton(s). Two of the ethylene protons could not be seen as their shift coincide with the water signal.

further modifying the *N*-methyl analogues (7, 8, and 9). We thus synthesized and evaluated compounds 10 to 16, modified on the 6-membered ring of benzotriazole. Our docking studies suggested that molecules 10, 11, and 12 could form a H-bond with heme propionate through their aniline. The most potent molecule remained the N<sub>2</sub> analogue 10. The affinity of these compounds was not significantly different from their precursors, but their lower  $c \log D_{7,4}$  value resulted in a better LLE (Table 2). Of note, the replacement of benzotriazole by 4-*aza*-benzotriazole in compounds 13, 14, and 15 did not result in better activities.

Next, we investigated the impact of bulkier N-chain substituents in N1 and N3 positions of benzotriazole (compounds 17 to 40). As the  $N_2$ -substitution was poorly available for alkylation, which only occurred through direct methylation of benzotriazole, we investigated only N1 and N3 substitutions. Lipophilic substituents such as trifluoroethyl (17 and 18) and ethyl-cyclopropyl (19 and 20) yielded potent compounds, as expected from their higher  $c \log D_{7.4}$ . We quickly noticed that N<sub>1</sub>-substitution was generally more favorable than N<sub>3</sub> for the activity. Interestingly, this trend was not apparent for the smaller methyl group as compounds 7, 8, and 9 exhibit the same activity. For example, N<sub>1</sub>-substitution with a trifluoroethyl  $([8, IC_{50}(enz): 1.9 \,\mu M/IC_{50}(cell)_{l}: 0.19 \,\mu M]$  resulted in a 3- to 7-fold increase in activity compared to N<sub>3</sub>-substitution of the same moiety [17, IC<sub>50</sub>(enz): 15.0  $\mu$ M/IC<sub>50</sub>(cell): 0.44  $\mu$ M]. However, N-substitution by the less lipophilic acetamide moiety

(21, 22) resulted in less potent TDO2 inhibitors with similar affinity, although the N<sub>1</sub>-substituted compound 22 appeared 2fold more potent in cells than its isomer. Surprisingly, their hydrolyzed counterparts 23 and 24 displayed a loss of activity in both cellular and biochemical assays, suggesting that a low membrane permeability was not the only reason that could account for their poor activity. Compounds 25 and 26, displaying a 2-(dimethylamino)ethyl moiety, shared a similar potency, perhaps due to the more flexible nature of their Nsubstituent. A further increase in the N-substituent size with physiologically nonionized moieties (27 to 32) led to a systematic loss of activity and an increasing gap between cellular and enzymatic potencies. Interestingly, the introduction of longer N-substituents with H-bond donors such as piperidine (33 and 34) and pyrrolidine (35 and 36) led to a better activity for N<sub>3</sub>-substituted compounds. Of note, these compounds had the best LLE values of all the studied compounds due to their predominantly protonated state at physiological pH, resulting in low  $c \log D_{7,4}$  values.

We also investigated a substitution of the indole ring H<sub>6</sub> to fluorine, with the synthesis of compounds **8a** and **37a**. As expected from previous results,<sup>40</sup> substitution with fluorine in this position of the indole was beneficial for the activity, and compound **37a** demonstrated a fivefold increase in activity in both cellular and enzymatic assays [IC<sub>50</sub>(enz): 5.2  $\mu$ M/ IC<sub>50</sub>(cell): 0.23  $\mu$ M] compared to **37** [IC<sub>50</sub>(enz): 24.9  $\mu$ M/

IC<sub>50</sub>(cell): 0.74  $\mu$ M]. The same gain in activity occurred for 8a in cells [IC<sub>50</sub>(enz): 1.1  $\mu$ M, IC<sub>50</sub>(cell): 0.02  $\mu$ M] compared to 8.

STD-NMR Study of the Interaction between the Active Site and the Inhibitor. To better understand the observed SARs and notably how the nature and positioning of the lateral N-sidechain influenced the binding of the inhibitor inside the active pocket, we performed epitope mappings on several couples of regioisomers using saturation transfer difference NMR (STD-NMR). In STD-NMR, selective saturation of *h*TDO2 in solution is transferred to the bound ligand through intermolecular nuclear Overhauser effects (NOEs) between protons of the inhibitor and the active site's residues. Consequently, the ligand's protons interacting more closely display intense STD signals, while a decrease in STD intensity correlates with lower interaction.<sup>41</sup> Due to solubility issues, we focused our investigation on compounds bearing solubilizing moieties to attain a high compound concentration (up to 200  $\mu$ M) in aqueous buffer, suitable for the present evaluation. We mapped the epitopes of regioisomers 25-26, 35-36, and 31-32 (Figure 3). Epitope mapping of all six compounds suggested that N<sub>3</sub>-substitutions led to an improper position of the indole ring compared to N<sub>1</sub>-substitutions (data regarding compounds 25-26 and 35-36 can be found in Supporting Information, Figure S2). Indeed, as pictured in Figure 3, epitope mapping on the different compounds showed that the indole ring displays the highest saturation transfer for protons  $H_{5}$ ,  $H_{6}$ , and  $H_{7}$ (around 100%). Concurrently, cocrystallographic structures of hTDO2 with L-Trp show that this part of the indole tightly interacts with a cluster of several hydrophobic residues involving L120, F5, Y24, Y27, and L28.<sup>39</sup> In the case of L-Trp, the indole  $H_2$  position is located very close to the heme.<sup>39</sup> Similarly, 26, 36, and **32** all exhibited a saturation transfer of around 90% for  $H_2$ . However, we observed that the less active N3-substituted analogues 25, 35, and 31 systematically displayed a 20 to 30% decrease in saturation transfer for H<sub>2</sub> compared to their regioisomers substituted in N1. Overall, these results suggest a shift in the position of the indole core for N<sub>3</sub>-substituted compounds, resulting in misplacement of H<sub>2</sub>. This apparent shift of the indole position could, at least in part, explain the difference in activity between N1- and N3-substituted compounds.

Interestingly, the sidechain of the different isomers always showed a similar and low saturation transfer compared to the aromatic protons. Therefore, the nature of the sidechains does not appear to be useful for the interaction with hTDO2, which opens the door to further modulation to fine-tune this family of inhibitor physicochemical properties.

**Molecular Docking.** Our molecular docking studies suggested two main hindrances for the accommodation of different ligands in the active site of *h*TDO2. The first identified hindrance was Arg144, as a shift in its sidechain was required for the binding of the inhibitors. Displacement of this sidechain increased with the size of the N-substituent and was more important for N<sub>3</sub> regioisomers. We noticed that wider Arg144 shifts correlated with lower enzymatic activity. The second hindrance was the flexible 338–346 loop, suggesting that an induced-fit-type binding was perhaps needed for larger compounds. It was previously described that disordering of this loop allows NFK to diffuse out and enables debinding of a new L-Trp molecule.<sup>39</sup> Its mobility was particularly noticeable when *h*TDO2 was cocrystallized with an NLG919-type analogue, widely opening the active site.<sup>28</sup>

Regioisomers displayed a similar binding pose, as shown in Figure 4, for analogues 17  $[IC_{50}(enz): 15.0 \,\mu\text{M/IC}_{50}(cell): 0.44$ 



**Figure 4.** Molecular modeling of regioisomers **17** (pink) and **18** (green), bearing a trifluoroethyl from the front (a) and the side (b). (PDB code: 5TI9)<sup>39</sup>

 $\mu$ M] and **18** [IC<sub>50</sub>(enz): 1.9  $\mu$ M, IC<sub>50</sub>(cell): 0.19  $\mu$ M] with a particularly noticeable affinity difference. However, docking of N<sub>1</sub>-substituted compounds resulted in a more favorable geometry for the indole core placement. Indeed, N<sub>3</sub>-substituted compounds display a shift of the indole core from the position naturally adopted by the indole of L-Trp, with a shift of C2, disturbing the interaction with the aromatic hotspots. Regarding compounds **33** to **40**), our docking results suggested a possible interaction with the propionate of the heme or with a H-acceptor sidechain (such as Ser345) from the mobile loop. This interaction N<sub>3</sub> analogues.

**Phase I Metabolic Stability and Electrophilic Reactivity.** The potential reactivity of **LM10** as a Michael acceptor was assessed with a thiol-trapping assay using cysteamine as the S-nucleophile and compared to that of the unsubstituted benzotriazole **6**.<sup>42–44</sup> Following incubation with cysteamine, the <sup>1</sup>H NMR spectrum of **LM10** quickly changed to reveal the apparition of several new aromatic signals. In contrast, the compound **6** spectrum stayed the same (Figure 5). Accordingly, LC/MS analysis of **LM10** ( $[M + H]^+ = 230.1$ ) confirmed the apparition of a secondary product ( $[M + H]^+ = 305.1$ ) upon cysteamine addition while **6** was not impacted.

Then, we evaluated the microsomal half-life of LM10 and several representative benzotriazoles (Table 3). The recovery percentage of LM10 after 24 h remained close to the initial concentration. Regarding the present series, we noticed that the nature of the N-substituent was determinant for the recovery rate of the compounds. The presence of fluorine on the indole did not impact the microsomal stability in compounds 8a and 37a compared to their nonfluorinated analogues. Furthermore, in isomers 37 and 38, the localization of the N-substituent did not impact the microsomal stability.



**Figure 5.** Aromatic regions of <sup>1</sup>H NMR in DMSO- $d_6$  spectra of 1 mM of **LM10** (a) and **6** (c) before and after (b,d) addition of 10 equivalents of cysteamine. Spectra were recorded 30 min after the addition of cysteamine. Spectrum (b) (**LM10** + cysteamine) shows several new aromatic signals, while proton integrations of compound **6** remain unchanged but displayed a slight shift in the presence of cysteamine.

Overall, these data demonstrate that the structural rigidification results in a scaffold that does not exhibit Michael acceptor behavior. Nonetheless, the microsomal stability studies contrast our initial idea that this template rigidification would necessarily lead to more metabolically stable compounds. Indeed, the present series display considerable heterogeneity in metabolic stability, which appears heavily dependent on the nature of the N-substituent.

**Chemistry.** The target compounds were synthesized by Suzuki–Miyaura cross-coupling between halogenated benzo-triazole analogues and the appropriate indole boronic ester.

Compounds 1-5 were obtained following the reaction between brominated isoquinolines or quinazolines and 41. The synthesis of 680C91-type analogues was performed using the same strategy, starting from 3 in the cross-coupling reactions (Scheme 1).

Several routes were attempted to synthesize compound 6, bearing an unsubstituted benzotriazole, leading to the synthetic pathway depicted in Scheme 2. We used *p*-methoxybenzyl (PMB) as a protecting group for 5-bromo-benzotriazole, which led to the formation of two regioisomers. Then, we performed the cross-coupling using the MIDA boronate 43 following a procedure adapted from Knapp et al.<sup>45</sup> The MIDA boronate

allows a continuous release of the boronic acid over several hours. This unique capacity enabled us to obtain the crosscoupled product with an almost quantitative yield. The crosscoupled product was first deprotected from its phenylsulfonyl ( $SO_2Ph$ ) using KOH, after which the PMB was removed in pure trifluoroacetic acid (TFA) at reflux temperature for 16 h to afford **6**. We noted that no degradation of the scaffold occurred despite the harsh basic and acidic conditions of both deprotection.

Two different routes, depicted in Scheme 3 and Scheme 4, were used to synthesize N-substituted benzotriazoles, either by direct alkylation of commercially available 5-halo-benzotriazole (Scheme 3) or by nucleophilic aromatic substitution starting from bromofluoronitrobenzene derivatives (Scheme 4), followed by reduction of the aromatic nitro group and treatment of the resulting compounds by sodium nitrite in acetic acid to get the final alkylated benzotriazoles (7b–23b and 17d–40d). To assess the substitution influence on the 6-membered ring, we took advantage of a highly electron-rich position between the halogen and the carbon between the two cycles to perform nitration using KNO<sub>3</sub> in H<sub>2</sub>SO<sub>4</sub>. A reduction using Fe<sub>(s)</sub>/NH<sub>4</sub>Cl was then performed to reduce the aromatic nitro group (Scheme 5).

Table 3. Microsomal Stabilities (Half-Lives) ofRepresentative Compounds $^a$ 

N°	Nx	R1	Microsomal half-life(min.)
LM10	1	/	> 1440
6	/	н	62
8	N <sub>3</sub>	∗CH <sub>3</sub>	24
8a	N <sub>3</sub>	∗CH <sub>3</sub>	22
18	N <sub>1</sub>	* CF3	45
26	N_1	*N	43
35	N <sub>3</sub>	*NH	> 1440
37	N <sub>3</sub>		> 1440
37a	N <sub>3</sub>	*N	> 1440
38	N <sub>1</sub>		> 1440

<sup>*a*</sup>The stability values were obtained in duplicates in two separate experiments. Graphs of stability curves can be found in Supporting Information (Figure S3).

Scheme 1. General Synthetic Procedure for Compounds  $1-5^a$ 



<sup>a</sup>Reagents and conditions: (a) **3** (1-(phenylsulfonyl)-3-(4,4,5,5-tetramethyl-1,3,2-dioxaborola*N*-2-yl)-1*H*-indole **42** (1.2 equiv), 5 mol % Pd(dppf)Cl<sub>2</sub>, 10 mol % SPhos,  $K_3PO_4$  (2.0 equiv), dioxane/ $H_2O$  (5:1), 60 °C, 3–6 h, under  $N_2$ . (b) KOH 2.5 M in MeOH/ $H_2O$  (1:1), reflux 30 min to 48 h.

The halogenated benzotriazoles (7b-23b and 17d-40d) were reacted with indole derivatives bearing a pinacol or MIDA boronate in the 3-position and a SO<sub>2</sub>Ph or a *tert*-butyloxycarbonyl (Boc) as a protecting group to afford the cross-coupled intermediates (Scheme 6). Finally, the target compounds 7-40 were eventually obtained after appropriate deprotection.

# CONCLUSIONS

In this work, we designed a series of original TDO2 inhibitors, devoid of the potentially problematic vinyl linker of the pubs.acs.org/jmc

reference compound LM10. We evaluated these compounds through a SAR study completed by STD epitope mapping and stability evaluation. These compounds present a wide range of lipophilicity and various chemical characteristics, showing the possibility of fine-tuning these inhibitors' molecular and pharmacokinetic characteristics for potential ADME purposes. Compared to existing potent TDO2 inhibitors (Figure 6a), the present series shows similar or slightly better (8a) cellular affinities, up to nanomolar range (Figure 6b). Previously, we reported that the enzyme seemed to be susceptible to small modifications of the inhibitor. However, we bring nuance to that affirmation, as TDO2 appears to allow bulkier substitutions for this series of compounds.

Interestingly, this series of compounds showed a systematically better activity on cells than on the isolated enzyme. Such a difference was previously observed for other indole-based patented TDO2 inhibitors.<sup>33,46</sup> Moreover, other previous works preferred focusing on cellular activities and did not include enzymatic inhibition values (i.e., preclinical compound **PF06845102/EOS200809**, evaluated here in the biochemical assay, Figure 6).<sup>25,27,28</sup> Different parameters could account for this discrepancy. First, the enzymatic assay that uses ascorbate, methylene blue, and catalase to maintain a reduced state could mimic poorly the physiological protein environment.<sup>47</sup> Furthermore, some inhibitors could also bind to the apo form of the protein, inhibiting its activity in a way that the enzymatic assay would not detect.

Finally, the isoquinoline and quinazoline derivatives reported in this work (1-5) also constitute attractive and highly functionalizable scaffolds for the future development of TDO2 inhibitors.

# EXPERIMENTAL SECTION

**Chemistry.** The following section comprises the general synthetic procedures used for the synthesis of compounds reported in this manuscript. Unless stated otherwise, reactions were performed under atmospheric pressure. Detailed experimental information and spectroscopic data of synthesized compounds are supplied in the Supporting Information. LC−MS analysis was performed on an Agilent (1100 series) HPLC single quadrupole (InfinityLab, ESI+) system equipped with a Kinetex 5  $\mu$ m EVO C18 (150 mm × 4,6 mm) using a gradient of distilled water + 0.1% TFA (buffer A) and acetonitrile + 0.1% TFA (buffer B). HPLC conditions for purity analysis were 5% B + 95% A at a flow rate of 1 mL/min, followed by an increase of solution B to 80% in 15 min (5%/min). The wavelengths for UV detection were 254 and 210 nm. HPLC purities of final compounds that underwent biological assessment were ≥95%.

The N-substituted benzotriazoles (18d-40d) were synthesized using the following three-step general procedure. (A) To a 0.5 M solution of 4-bromo-1-fluoro-2-nitrobenzene or 4-bromo-2-fluoro-1nitrobenzene (1 equiv) in ethanol was added the primary amine (1.5 equiv) and triethylamine (2 equiv). The resulting mixture was stirred under reflux until complete conversion (30 min to 16 h), before being evaporated in vacuo. The residue was extracted using EtOAc and water, and the organic layer was dried with Na2SO4 and concentrated to afford the desired compound, used in the next step without further purification (unless stated otherwise). (B) The nitro group of Nsubstituted 4-bromo-2-nitroaniline or 5-bromo-2-nitroaniline (1 equiv) obtained in step A was reduced into an amine using iron powder (10 equiv) in a mixture of NH<sub>4</sub>Cl<sub>sat</sub>./EtOH (1:4) stirred under reflux for 30 min to 16 h. Upon complete conversion, the reaction mixture was filtrated over a celite pad, washed with EtOAc. After extraction, the organic layer was dried over Na2SO4 and concentrated in vacuo. Unless stated otherwise, the obtained residue was used for step C without further purification. (C) The cyclization of mono-N-substituted 4bromobenzene-1, 2-diamine or 5-bromobenzene-1, 2-diamine was

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Scheme 2. Synthesis of Compound 5-(1H-Indol-3-yl)-1H-benzotriazole (6)<sup>a</sup>



<sup>a</sup>Reagents and conditions: (a) 5 mol % Pd(dppf)Cl<sub>2</sub>, 10 mol % SPhos,  $K_3PO_4$  (2.0 equiv), dioxane/H<sub>2</sub>O (5:1), 60 °C, 6 h, under N<sub>2</sub>. (b) KOH 2.5 M in H<sub>2</sub>O/MeOH (1:1), reflux, 1 h. (c) 100% TFA, reflux, 16 h.





<sup>a</sup>Reagents and conditions: (a) Me<sub>2</sub>SO<sub>4</sub> (1.6 equiv), NaOH<sub>aq</sub> 2 M, r.t., 1 h 30 (b) ClCH<sub>2</sub>CN (1.5 equiv), Et<sub>3</sub>N, DMF, reflux, 16 h.

Scheme 4. Synthetic Route for N-Substituted 5-Bromobenzotriazoles and 6-Bromobenzotriazoles (17d-40d)<sup>a</sup>

$Br + F \xrightarrow{NO_2} \xrightarrow{a} Br + F$	X	NO <sub>2</sub> N <sup>R</sup>	Br		Br <sub>6</sub>	∑ <sup>N</sup> N N R
R:	<u>5-Br</u>	<u>4-Br</u>	<u>5-Br</u>	<u>4-Br</u>	<u>6-Br</u>	<u>5-Br</u>
2,2,2,-trifluoroethyl	17b	18b	18c	18c	18d	18d
cyclopropylmethyl	19b	20b	19c	20c	19d	20d
2-(dimethylamino)ethyl	25b	26b	25c	26c	25d	26d
(tetrahydrofuran-2-yl)methyl	27b	28b	27c	28c	27d	28d
(tetrahydrofuran-3-yl)methyl	29b	30b	29c	30c	29d	30d
2-morpholinoethyl	31b	32b	31c	32c	31d	32d
tert-butyl 4-ethylpiperidine-1-carboxylate	33b	34b	33c	34c	33d	34d
tert-butyl 3-ethylpyrrolidine-1-carboxylate	35b	36b	35c	36c	35d	36d
tert-butyl 4-ethylpiperazine-1-carboxylate	37b	38b	37c	38c	37d	38d
butane-1,2-diol	39b	40b	39c	40c	39d	40d

<sup>*a*</sup>Reagents and conditions: (a) primary amine derivative,  $Et_3N$  (1.3 equiv), EtOH or THF, r.t. or reflux, 30 min to overnight; (b)  $Fe_{(s)}$  (10 equiv),  $EtOH/H_2O$  (4:1), reflux, 30 min to overnight; (c)  $NaNO_2$  (2.5 equiv), glacial acetic acid, sonication, r.t., 1 h.

performed using  $NaNO_2$  in acetic acid using a sonication bath for 30 min to 1 h. The reaction mixture was then poured onto ice water and extracted with EtOAc. The combined organic layers were washed with saturated  $Na_2CO_3$  solution and brine, dried with  $Na_2SO_4$ , and concentrated *in vacuo*. The N-substituted benzotriazole was purified using flash chromatography on silica gel with an EtOAc/cyclohexane gradient.

General Procedures for Nitration (D) of Chloro-methylbenzotriazoles. (D) 2 mmol of the appropriate chloro-methylbenzotriazole (7b–9b) was dissolved in 3.5 mL of concentrated  $H_2SO_4$ . To this solution, 6.5 mmol KNO<sub>3</sub> in concentrated  $H_2SO_4$  was added dropwise. The resulting mixture was agitated for 1 h 30 min at 50 °C. After cooling down to room temperature, the reaction mixture was poured on ice, resulting in precipitate formation (10b–12b). The obtained powder was washed with water until neutrality. In accordance

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Scheme 5. Synthetic Route for Nitration and Reduction to Anilines of Chloro-methyl-benzotriazoles<sup>a</sup>



<sup>a</sup>Reagents and conditions: (a) KNO<sub>3</sub> (3.0 equiv) in H<sub>2</sub>SO<sub>4</sub>, 50 °C, 1 h 30 (b) Fe<sub>(s)</sub> (10.0 equiv), EtOH/H<sub>2</sub>O (4:1), reflux, 30 min.

## Scheme 6. General Synthetic Procedure for Cross-Coupling and Deprotection of 6-(1H-Indol-3-yl)-benzotriazole Derivatives<sup>a</sup>



<sup>*a*</sup>Reagents and conditions: (a) boronic ester derivative (**41**, **42**, or **43**) (1.2–1.5 equiv), 5 mol % Pd(dppf)Cl<sub>2</sub>, 10 mol % SPhos, K<sub>3</sub>PO<sub>4</sub> (2.0 equiv), dioxane/H<sub>2</sub>O (5:1), 60 °C, 3–6 h, under N<sub>2</sub>. (b) HCl 4.0 M in MeOH/H<sub>2</sub>O (1:1), reflux, 30 min to 5 h for Boc deprotection, (c) KOH 2.5 M in MeOH/H<sub>2</sub>O (1:1) or 1 M TBAF in THF, reflux 30 min to 48 h. for SO<sub>2</sub>Ph deprotection.

with the literature, nitration of 5-chloro-2-methyl-2*H*-benzo[*d*][1,2,3]-triazole (7**b**) led to the formation of a second dinitrated product, 5-bromo-2-methyl-4,6-dinitro-2*H*-benzo[*d*][1,2,3]triazole, in addition to the mononitrated 5-chloro-2-methyl-4-nitro-2*H*-benzo[d][1,2,3]-triazole (10**b**).<sup>49</sup> The general reduction procedure (C) was used to reduce the nitrated chloro-methyl-benzotriazoles into *N*-methylbenzo-triazol-4-amines (10**c**-12**c**).

General Procedures for the Suzuki-Miyaura Cross-Coupling Reaction. Prior to the coupling procedures, the solvent 1,4-dioxane and K<sub>3</sub>PO<sub>4</sub> solution in water were degassed by atmosphere exchange with nitrogen in a sonication bath for 45 min. The cross-coupling procedure was adapted from Knapp et al.45 We found that the use of Pd(dppf)Cl<sub>2</sub> as a catalyst significantly increased the yield as compared to  $Pd(OAc)_2$ . (E) The coupling procedure was adapted for the use of a pinacol boronic ester (41 and 42). Aryl-halide (1 mmol, 1 equiv) and the boronic ester (1.6 equiv), Pd(dppf)Cl<sub>2</sub> (5 mol %) and SPhos (10 mol %), were introduced into a 25 mL two-neck round-bottom flask under an inert atmosphere. Then, 10 mL of degassed dioxane was added, and the reaction mixture was left under agitation at r.t. for 10 min until dissolution of reactants. Finally, 2 mL of degassed distilled water containing  $K_3PO_4$  (3.5 mmol) was added to the media, and the reaction mixture was stirred for 3 to 16 h at 70 °C. When the conversion was complete, the mixture was cooled to room temperature, diluted with 1 N NaOH (10 mL), and extracted twice with 10 mL of EtOAc. The

combined organic fractions were dried over  $Na_2SO_4$  and concentrated *in vacuo*. The obtained residue was purified by flash chromatography on silica gel with the appropriate gradient of EtOAc in cyclohexane to afford the cross-coupled protected intermediate. (F) A coupling procedure was adapted for the use of a MIDA boronate (43). The general procedure (E) was slightly adapted when couplings were performed using MIDA boronate 43. In this modified procedure (F), 1.2 equiv of boronate and 7 equiv of  $K_3PO_4$  were used.

General Phenylsulfonyl ( $SO_2Ph$ ) Deprotection Procedures of Cross-Coupled Products. Intermediates obtained using procedures (F) and (E), bearing an SO<sub>2</sub>Ph protecting group, were deprotected using deprotection procedures (G) or (H), while Boc deprotection was performed using the deprotection procedure (I).

**Deprotection Procedure G.** 0.5 mmol of starting compound was dissolved in 4 mL of a 1:1 solution of MeOH/H<sub>2</sub>O containing 2.5 M of KOH. The resulting mixture was refluxed until completion of the reaction (30 min to 48 h) to afford compounds 1-20, 23-27, and 31-40.

**Deprotection Procedure H.** 0.5 mmol of starting compound was dissolved in 4 mL of a 1 M solution of TBAF in THF. The resulting mixture was refluxed until completion of the reaction (2-48 h) to afford compounds 21-22 and 28-30.

**Deprotection Procedure I.** 0.5 mmol of starting compound was dissolved in 4 mL of a 1:1 solution of MeOH/H<sub>2</sub>O containing 4 M HCl.



Figure 6. Examples of potent TDO2 inhibitors (a) and representative examples of the series developed in the present work (b) (8a and 37a). <sup>a</sup>log  $D_{7,4}$  values were calculated using Chemicalize. <sup>b</sup>Compound 21 and its inhibition values are from Pei et al. 2018.<sup>48</sup> <sup>c</sup>Compounds 20 and 30 and their inhibition values are from Parr et al.<sup>28</sup> <sup>d</sup>Cellular potency of PF06845102/EOS200809 is from Schramme et al. 2020.<sup>25</sup> <sup>e</sup>Biochemical IC<sub>50</sub> was measured in the present work.

The resulting mixture was refluxed until completion of the reaction (30 min to 48 h) to afford compounds **8a**, **37a**, and **17–18**.

**Molecular Docking.** Molecular docking jobs were performed with AutodockVina 1.1.2,<sup>50</sup> using the disclosed tryptophan 2,3-dioxygenase structure (PDB ID: 5TI9)<sup>39</sup> with the orthosteric binding site occupied by natural ligand tryptophan, heme, and dioxygen. This structure was chosen as it displayed the full 338–346 loop that is part of this binding site. Hydrogens and charges were added, and then, tryptophan and dioxygen were removed, centering the searching box 15 Å around this region. During the process, sidechain flexibility was allowed for several residues of the binding site and later on only for Arg144 that appeared critical. Binding poses were analyzed for their binding mode and assessed in comparison with the crystallized analogue and with L-Trp.

Protein Expression and Purification. The sequence of the truncated form of *h*TDO2 in which 1–17 and 389–406 fragments were deleted (His6Thrb-TDO2tr),<sup>23</sup> described by Batabyal and Yeh, was inserted in a pET-28 plasmid, ordered from Genecust. The recombinant plasmid was transferred into the Escherichia coli strain BL21 (DE3) (Rosetta). The transformant was selected from a single colony on a LB kanamycin agar plate and was used to inoculate in a LB medium (50  $\mu$ g/mL kanamycin and 34  $\mu$ g/mL chloramphenicol) and was cultured at 37 °C until an optical density of 0.6 was reached. The expression of hTDO2 was induced by isopropyl 1-thio  $\beta$  galactopyranoside (IPTG), with a final concentration of 1 mM. An aliquot of hemin in NaOH 0.5 M, with a final concentration of  $8-10 \ \mu M$ , was added to the culture. The culture was grown at 20 °C for 20 h. Cells were harvested by centrifugation at 5000 rpm, 4 °C for 25 min. Cell pellets were resuspended in a lysis buffer (Tris-HCl 50 mM, pH 8.5, MgCl<sub>2</sub> 10 mM, NaCl 300 mM, imidazole 5 mM, and glycerol 10%) supplemented with protease inhibitors (Roche) and then disrupted by sonication, followed by centrifugation at 4 °C and 10,000 rpm for 30 min. The supernatant was collected, and to 1  $\mu$ L of  $\beta$ -mercaptoethanol per mL of the supernatant was added, before loading onto 1 mL Histrap FF-crude columns (GE Healthcare) according to the manufacturer's instructions. Protein concentrations were measured using the Bradford method with the Biorad protein assay kit, and sample homogeneity was assessed using sulfate-polyacrylamide gel electrophoresis with Coomassie brilliant blue as a staining agent. Purified TDO2tr was then dialyzed overnight in a pH 7.6 buffer containing 50

mM sodium phosphate, 100 mM NaCl, 20% glycerol, and 10 mM L-Trp. To ensure of the quality and proper folding of the purified enzyme, nano differential scanning fluorimetry experiments were performed on a Tycho NT.6 device (NanoTemper Technologies). According to the standard manufacturer's procedures, samples were poured into capillaries and heated up to 95 °C in 3 min while following fluorescence emission at 330 and 350 nm. Melting temperatures were extracted from the derivative of the 350/330 nm fluorescence ratios upon increasing temperature.

**Nuclear Magnetic Resonance.** All experiments were performed at 277 K on a Bruker Ascend AVANCE III 600 MHz system equipped with a broadband cryoprobe (Bruker). For 1D STD studies, samples were prepared at 200  $\mu$ M in PBS with 5% deuterated methanol. The concentration of hTDO2<sub>tr</sub> was 15  $\mu$ M. Ligand binding was detected using an STD stddiffesgp.3 sequence with a 2 s saturation time. Water signal suppression was achieved using an excitation-sculpting scheme, and a 50 ms spinlock was used to suppress protein background signals. For each experiment, 2048 scans were collected for on- and off-resonance experiments (respectively at 0 and 40 ppm) to achieve a sufficient signal to noise ratio for epitope mapping.

**Enzymatic Assay.** All the graphs were obtained with Graph-PadPrism 8 software (San Diego, CA). The UV spectra were recorded at room temperature on a Spectramax M2E spectrophotometer (Molecular Devices, LLC, Sunnyvale, CA) in 96-well flat bottom plates. The assay was performed in a pH 6.5 potassium phosphate buffer (100 mM) at 37 °C. The final concentrations in each well were 100  $\mu$ M L-Trp, 20 mM L-ascorbate, 10  $\mu$ M methylene blue, 150 nM catalase, and 3% of DMSO containing the inhibitor and (optionally) 0.01% v/v of Triton X-100. The assay was initiated by adding 15  $\mu$ L of buffer containing *h*TDO2<sub>tr</sub> (no L-Trp, no DMSO) to 85  $\mu$ L of buffer (with L-Trp and the tested inhibitor concentration in DMSO). After 15 min, 20  $\mu$ L of 30% m/v of trichloroacetic acid in water was added, and the plates were heated at 65 °C for 15 min. Then, 100  $\mu$ L of a 2% m/v *p*-dimethylbenzaldehyde solution in pure acetic acid was added to each well. After 10 min, absorbance reading was performed at 490 nM.

**Cellular Assays.** The assay was performed in 96-well flat bottom plates seeded with  $1 \times 10^5$  cells (P815B-*h*TDO, clone 19) in a final volume of 200  $\mu$ L of Iscove's modified Dulbecco medium (GIBCO-Thermo Fisher Scientific; which contains 80  $\mu$ M L-Trp) supplemented

with 2% FCS in the presence of the TDO inhibitor at different concentrations (1–25,000 nM) for 7 h when substrate consumption was below 25%. Cells were centrifuged 10 min at 300 rcf, after which 60  $\mu$ L of supernatant was collected and mixed with 60  $\mu$ L of 12% (wt/vol) trichloroacetic acid. After centrifugation at 4 °C at 23,000 rcf, 70  $\mu$ L of supernatant was diluted with 70  $\mu$ L of Milli-Q H<sub>2</sub>O. The resulting solution was used to quantify L-Trp and Kyn concentrations by ultraperformance liquid chromatography (UPLC) based on the retention time and UV absorption. Briefly, samples were injected into the Kinetex 2.6  $\mu$ m EVO C18 100A (2,1 × 50) column (Phenomenex) and were eluted using a 1,7 min linear gradient of acetonitrile in water (5–50%) containing 0.1% trifluoroacetic acid at a flow rate of 0,6 mL/min. The column eluent was monitored with a UV detector at 280 and 360 nm to detect L-Trp and Kyn, respectively.

For hIDO counter-screening (P815B-hIDO, clone 6), the same protocol was applied, but the plates were seeded with  $2 \times 10^5$  cells and incubated for 24 h in order to achieve a similar substrate consumption.

*In Cellulo* Assessment of Toxicity. A solution  $(100 \ \mu L)$  containing 80  $\mu$ L of PBS, 20  $\mu$ L of PMS, and 1  $\mu$ L of MTS was added to wells containing 1 × 10<sup>5</sup> cells in 100  $\mu$ L of DMEM. After 2–4 h of incubation with MTS and PMS at 37 °C, absorbance at 490 nm was measured.

Microsomal Stability Assay. Liver microsomes (20 mg protein/ mL), NADPH regenerating system solutions A and B, and 10 mM stock compound solution (100% DMSO) were prepared. The reaction mixture finally contains 713  $\mu$ L of purified water, 200  $\mu$ L of 0.5 M potassium phosphate pH 7.4, 50 µL of NADPH regenerating system solution A (BD Biosciences Cat. no. 451220), 10 µL of NADPH regenerating system solution B (BD Biosciences Cat. no. 451200), and 2  $\mu$ L of the compound stock solution (10  $\mu$ M final concentration). A control experiment was realized for each compound by substituting NADPH regenerating solutions A and B for 60  $\mu$ l of purified water. The reaction mixture is warmed to 37 °C for 5 min in a water bath, and the reaction is initiated by the addition of 25  $\mu$ L of liver microsomes (0.5 mg protein/mL final concentration). At different time points (0 min; 60 min; 180 min; 360 min; 24 h), 100  $\mu$ L is withdrawn and added to 400  $\mu$ L of cold acetonitrile on ice. Then, the mixtures are centrifuged at 13,000 rpm for 5 min at 4 °C. Finally, 450  $\mu$ L of the supernatant is recovered, evaporated using SpeedVac, and evaluated by UPLC. 7-Ethoxycoumarine was used as a positive control.

#### ASSOCIATED CONTENT

## **Supporting Information**

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jmedchem.1c00323.

Atomic coordinates for compound 17 inside hTDO2 cavity (PDB)

Atomic coordinates for compound 18 inside hTDO2 cavity (PDB)

Atomic coordinates for compound **6** inside *h*TDO2 cavity (PDB)

Inhibitory affinities; evaluation of influence of compound 6; epitope mapping; microsomal stability and half-life curves; enzymatic IC50 curves; cellular IC50 curves; HPLC traces; chemicals and reagents; synthesis of boronic esters; and synthesis of N-substituted benzo-triazoles (PDF)

Molecular formula strings of the synthesized compounds (CSV)

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# Notes

The authors declare no competing financial interest.

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# ABBREVIATIONS

ADME, absorption, distribution, metabolism, and excretion; AhR, aryl hydrocarbon receptor; GCN2, general control nonderepressible 2; IDO1, indoleamine-2,3-dioxygenase 1; KMO, kynurenine-3-monooxygenase; KP, tryptophan-kynurenine pathway; KYN, kynurenine; LE, ligand efficiency; LLE, lipophilic ligand efficiency; L-Trp, L-tryptophan; MDSCs, myeloid-derived suppressor cells; NK, natural killer cell; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; SMI, small-molecule inhibitor; STD, saturation transfer difference; TDO2, tryptophan 2,3-dioxygenase; Treg, regulatory T-cells

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