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Published in:

Proceedings of the National Academy of Sciences of the United States of America

DOI:

[10.1073/pnas.1113873109](https://doi.org/10.1073/pnas.1113873109)

Publication date:

2012

Document Version

Publisher's PDF, also known as Version of record

[Link to publication](#)

Citation for published version (HARVARD):

Pilotte, L, Larrieu, P, Stroobant, V, Colau, D, Dolušić, E, Frédérick, R, De Plaen, E, Uyttenhove, C, Wouters, J, Masereel, B & Van Den Eynde, BJ 2012, 'Reversal of tumoral immune resistance by inhibition of tryptophan 2,3-dioxygenase', *Proceedings of the National Academy of Sciences of the United States of America*, vol. 109, no. 7, pp. 2497-2502. <https://doi.org/10.1073/pnas.1113873109>

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Reversal of tumoral immune resistance by inhibition of tryptophan 2,3-dioxygenase

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Edited by Vincenzo Cerundolo, John Radcliffe Hospital, Oxford, United Kingdom, and accepted by the Editorial Board January 9, 2012 (received for review August 25, 2011)

Tryptophan catabolism mediated by indoleamine 2,3-dioxygenase (IDO1) is an important mechanism of peripheral immune tolerance contributing to tumoral immune resistance, and IDO1 inhibition is an active area of drug development. Tryptophan 2,3-dioxygenase (TDO) is an unrelated hepatic enzyme that also degrades tryptophan along the kynurenine pathway. Here, we show that enzymatically active TDO is expressed in a significant proportion of human tumors. In a preclinical model, TDO expression by tumors prevented their rejection by immunized mice. We developed a TDO inhibitor, which, upon systemic treatment, restored the ability of mice to reject TDO-expressing tumors. Our results describe a mechanism of tumoral immune resistance based on TDO expression and establish proof-of-concept for the use of TDO inhibitors in cancer therapy.

cancer immunotherapy | small molecule inhibitor | immunomodulation | immune suppression

Tryptophan 2,3-dioxygenase (TDO) is a homotetrameric heme-containing cytosolic enzyme encoded by gene *TDO2* and expressed at high levels in the liver. It catalyses the first and rate-limiting step of tryptophan degradation along the kynurenine pathway and thereby regulates systemic tryptophan levels. The same reaction can be catalyzed by another heme-containing cytosolic enzyme, named indoleamine 2,3-dioxygenase (IDO1), which has no sequence similarity with TDO, is not expressed in the liver, and is monomeric. IDO1 has been the focus of attention in recent years because of its immunosuppressive effects on T lymphocytes, resulting partly from tryptophan depletion and partly from direct effects of tryptophan catabolites (1–3). IDO1 is expressed constitutively in the placenta, where it plays a key role in feto-maternal tolerance (2), and in many tumors, where it contributes to tumoral resistance to immune rejection (4–7). IDO1 expression is also inducible in many cells, including dendritic cells, and appears to play a role in peripheral immune tolerance and the retro-control of immune responses (8). In contrast, little is known about the effect of TDO expression on the immune response. A recent report indicated that human cells transfected with TDO depleted tryptophan and thereby prevented both the growth of pathogens and the proliferation of allogeneic T lymphocytes (9). These results suggested that TDO might mediate immunosuppressive effects similar to those of IDO1. We set out to examine whether tumor cells express TDO and thereby inhibit T-cell-mediated immune responses.

Results

We first observed that many human tumor samples expressed gene *TDO2* as measured by real-time RT-PCR (Table 1). This was the case for 41% of bladder carcinomas, 50% of melanomas and 100% of hepatocarcinomas. To confirm the activity of TDO in tumor cells, we selected a series of human tumor cell lines that also expressed the *TDO2* mRNA. We incubated cells for 24 h in medium containing a known concentration of tryptophan, and measured by HPLC in the supernatant the concentration of

tryptophan and kynurenine, which is the main tryptophan catabolite (Table 2). HEK-293 cells transfected or not with human *TDO2* were used as positive and negative controls. We observed a clear activity in cell lines expressing more than one copy of *TDO2* mRNA per cell. Because some of these tumor lines also expressed IDO1, we used TDO inhibitor 680C91 (10) and IDO1 inhibitor 1-methyl-L-tryptophan (1MT) (4) to distinguish the activity of both enzymes in the cellular assay. In cell lines expressing TDO and not IDO1, tryptophan degradation was completely blocked by 680C91 and not by 1MT, as expected (Table 2). In cell lines expressing both enzymes, an additive effect of both inhibitors was observed (Table 2 and Fig. S1). The TDO activity of those lines and its direct inhibition by 680C91 were confirmed in an enzymatic assay performed on crude cell extracts, which is not dependent on the transport of the substrate and the inhibitor across the cell membrane (Table 2 and Fig. S2).

To determine whether TDO expression allows tumor cells to resist immune rejection by T cells, we used the P815 mouse tumor model (4). P815 tumor cells regularly produce tumors when injected intraperitoneally into naïve syngeneic DBA/2 mice, even though they are clearly immunogenic and express several tumor antigens recognized by cytolytic T lymphocytes (CTL). One of these antigens is encoded by cancer germ-line gene *P1A* and is the major target of the rejection response of mice immunized against P815 (11). Mice immunized against this antigen reject a challenge of P815 tumor cells injected in the peritoneal cavity (12). We transfected subline P815B, which does not express *TDO2*, with an expression plasmid containing the mouse *TDO2* cDNA. We selected transfected P815B mTDO clones 8 and 12, which degraded tryptophan at levels similar to human tumor lines (Table 2). We then immunized mice against the P1A antigen and injected them 4 wk later with an i.p. challenge of transfected P815 cells. As expected, most mice rejected the control P815B clone 1, which was transfected with an empty vector and did not express TDO (Fig. 1A). In contrast, most mice injected with TDO-expressing tumor cells developed progressive tumors and died. This was true for both TDO-transfected clones. The three clones produced progressive tumors in all naïve mice, but the TDO-expressing tumors grew somewhat faster (Fig. 1B). This might result from the occurrence of a primary immune response that retards the growth of the tumors and is abolished

Author contributions: L.P., P.L., J.W., B.M., and B.J.V.d.E. designed research; L.P., P.L., V.S., D.C., E.D., R.F., E.D.P., and C.U. performed research; V.S., D.C., E.D., R.F., J.W., and B.M. contributed new reagents/analytic tools; L.P., P.L., D.C., R.F., E.D.P., and B.J.V.d.E. analyzed data; and L.P., P.L., and B.J.V.d.E. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission. V.C. is a guest editor invited by the Editorial Board.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1113873109/-DCSupplemental.

Table 1. TDO2 expression in human samples

Sample type	TDO2-positive samples (no. positive/ no. tested)	TDO2 mRNA/cell in positive samples	
		Mean	Min–max
Tumors			
Bladder carcinoma	9/22	23.7	(2.6–125)
Hepatocarcinoma	7/7	791.7	(47.4–4,000)
Melanoma	10/20	5.6	(2.0–16.7)
Mesothelioma	2/4	8.5	(2.4–14.6)
Neuroblastoma	2/3	5.2	(2.1–8.4)
Sarcoma	1/5	5.9	(5.9)
Breast carcinoma	2/17	12.2	(7.8–16.7)
Leukemia	1/25	16.7	(16.7)
Renal cell carcinoma	1/7	88.4	(88.4)
Colorectal carcinoma	2/7	9.2	(4.8–13.6)
Head & neck carcinoma	1/9	9.0	(9.0)
Lung carcinoma	2/8	11.4	(3.6–19.2)
Brain tumor	2/10	4.7	(4.2–5.3)
Normal tissues			
Liver	3/3	876.3	(406.1–1515.7)
Skin	0/2	0.0	(0.0)
Bladder	0/2	0.1	(0.05–0.2)
Breast	0/3	0.1	(0.0–0.1)
Blood	0/2	0.0	(0.0)

Expression of *TDO2* mRNA was measured by quantitative RT-PCR. Each cDNA was performed at least in duplicate. *TDO2* expression was normalized to actin and the amount of mRNA molecules per cell was calculated. Tumor samples containing at least two molecules of *TDO2* mRNA per cell were considered positive. For normal tissues, means and min-max are provided for all samples.

with the TDO-expressing tumors. The intrinsic tumorigenicity of the three clones was similar, as indicated by their identical progression in irradiated mice (Fig. 1B). They also expressed identical levels of *P1A* mRNA, as measured by semiquantitative RT-PCR, and were equally lysed by P1A-specific CTL (Fig. 1C). These results were very similar to those we reported previously with IDO1-expressing tumor cells, and suggested that the progression of TDO-expressing tumors in P1A-immunized mice resulted from the ability of those tumors to prevent their rejection by T lymphocytes. To visualize this effect on T lymphocytes, we used H-2L^d/P1A tetramers to measure the number of P1A-specific CTL in the peritoneal cavity of immunized mice before and after the challenge with P815 tumor cells. Immunized mice contained detectable amounts of P1A-specific CTL, which further increased four days after challenge with P815B control cells (Fig. 1D). This increase was limited in mice challenged with TDO-expressing P815B mTDO cl12, suggesting that TDO expression reduces T lymphocyte proliferation locally.

The results outlined above indicated that pharmacological inhibition of TDO could combat tumoral immune resistance and promote tumor rejection. So far, several compounds were reported as TDO inhibitors (10, 13–15). One series, initially developed by Madge and Salter as combined TDO/serotonin reuptake inhibitors for depression therapy, is characterized by a (fluoro)indole scaffold substituted in the 3-position by a pyridinyl-vinyl side chain. Among these compounds, the analog 680C91 represents a very attracting compound, as it is endowed with a good TDO inhibition potency and is deprived of activity on serotonin reuptake (10). As a first step, we assessed bioavailability of 680C91 in mice by the oral route. After administration of 160 mg/kg/day in the drinking water up to 7 d, we measured plasma concentrations of 680C91 below 0.2 µg/mL (0.8 µM), indicating the poor bioavailability of the compound, presumably related to its poor solubility (Fig. 24). We thus started a medicinal chemistry program aimed at improving the aqueous

solubility and bioavailability in this series (16). This eventually led to the discovery of compound LM10, which is characterized by a 6-fluoro-indole substituted in the 3-position by a tetrazolyl-vinyl side chain and displays a good TDO inhibition ($K_i = 5.6 \mu\text{M}$) with a competitive inhibition profile (Fig. 24; ref. 16). LM10 does not inhibit IDO and has a high solubility and bioavailability (Fig. 24). The plasma concentration of LM10 after oral administration of 160 mg/kg/day was between 20 and 40 µg/mL (87–175 µM), a concentration about 40 times above the IC₅₀ measured in the cellular assay performed with the physiological concentration of plasma tryptophan (80 µM; Fig. 24; ref. 17).

To determine whether systemic treatment with LM10 was able to promote rejection of TDO-expressing tumors, we immunized and challenged mice as above and administered LM10 in the drinking water (Fig. 2B). We observed that systemic treatment of immunized mice with LM10 (160 mg/kg/day) prevented the growth of TDO-expressing P815 tumor cells. This was true for both TDO-transfected clones. Surprisingly, LM10 treatment also promoted better rejection of control clone P815B cl1, which does not express TDO. One explanation for this might be the expression of TDO by macrophages at the tumor site, as activation of macrophages by phorbol esters or intracellular infection was shown to induce TDO expression (18, 19). We confirmed expression of *TDO2* mRNA in F4/80⁺ macrophages isolated from the peritoneal cavity of both control mice and immunized mice carrying ascitic P815B cl1 tumor cells (Fig. S3).

Mice treated with LM10 did not show obvious signs of toxicity. Because TDO is mostly expressed in the liver, we evaluated liver damage at the end of the experiment as a first attempt to evaluate the safety of LM10 treatment. After more than 100 d of systemic administration of LM10, we found no significant difference between treated and untreated mice for their plasma level of hepatic enzymes alkaline phosphatase (ALAP), γ -glutamyltransferase (γ -GT), alanine aminotransferase (ALAT), and aspartate aminotransferase (ASAT) (Table 3). Those levels remained within the normal range, suggesting the lack of liver toxicity of prolonged TDO inhibition with LM10.

To determine whether the antitumor effects of LM10 resulted only from promoting tumor rejection or whether LM10 also had a direct effect on tumor cells, we injected immunodeficient *RAG2* knockout mice with TDO-expressing P815 cells and treated them with LM10 as above. Tumor growth was not affected by LM10 treatment in *RAG2* knockout mice, indicating that the effect of LM10 was dependent on the immune system in this model (Fig. 3).

Discussion

Cancer immunotherapy is emerging as a promising approach for cancer treatment. Various strategies to modulate antitumor responses have been tested for many years and some of them recently proved clinically useful or even gained FDA approval (20–26). However, it is also becoming clear that an important limitation of cancer immunotherapy results from the ability of some tumors to resist immune rejection (27). A variety of mechanisms can account for such resistance, only some of which are amenable to modulation to improve the efficacy of cancer immunotherapy. One such mechanism is based on tryptophan catabolism by IDO1, which is frequently expressed in tumors (4, 6). We previously observed in a preclinical model that rejection of IDO1-expressing tumors was promoted by systemic treatment with an IDO1 inhibitor (4). The search for IDO1 inhibitors that can be used clinically is ongoing (7, 28–30). We show here that tumors also use another means to degrade tryptophan and resist immune rejection: they express TDO, the tryptophan-degrading enzyme normally expressed almost exclusively in the liver. Interestingly, the immunosuppressive effect of liver-expressed TDO might contribute to the allograft tolerance usually observed after liver transplantation, as recently suggested (9).

Table 2. TDO expression and activity in tumor cell lines

Inhibitor	<i>TDO2</i> mRNA/cell*	<i>IDO1</i> mRNA/cell*	Kynurenine production (pmol kynurenine/hr/10 ⁶ cells)					
			Cellular assay [†]				Assay on crude extract [‡]	
			-	680C91	1MT	680C91+1MT	-	680C91
Human cell lines								
293-E	0.0	0.0	0	0	0	0		
293-E hTDO cl105	993.1	0.0	2,462	0	2,051	0	1,111 (±13)	61 (±9)
293-E hTDO cl119	1,231.1	0.0	3,743	94	3,333	105	544 (±14)	15 (±3)
HepG2 (hepatocarcinoma)	0.0	0.0	0	0	0	0		
Huh7 (hepatocarcinoma)	1.9	0.0	41	0	0	0		
LB159-CRC (colorectal carcinoma)	67.0	0.0	2,007	0	1,869	0		
SK-CO-11 (colorectal carcinoma)	0.5	0.0	0	0	0	0		
LB1317-SCCHN (head & neck carcinoma)	58.3	19.2	2,973	715	2,310	303	3,252 (±14)	277 (±19)
A172 (glioblastoma)	31.2	1.6	3,753	119	3,775	0	708 (±7)	18 (±5)
HTZ-349 (glioblastoma)	0.1	0.0	0	0	0	0		
U-87-MG (astrocytoma)	8.7	0.0	413	0	559	0		
SK-Mes-1 (lung carcinoma)	6.3	0.4	1,117	926	515	354		
LB2259-MEL (melanoma)	0.1	0.0	0	0	0	0		
MZ2-MEL (melanoma)	0.3	0.0	0	0	0	0		
MZ-CHA-3 (gall-bladder carcinoma)	4.5	35.9	534	366	217	144		
Murine cell lines								
Mastocytoma P815, subline P815B	0.0	0.0	ND	ND				
Transfected P815B cells:								
P815B cl1 (control)	0.0	0.0	0	0			0	
P815B mTDO cl8	129.6	0.0	4,857	103			2,330 (±32)	55 (±5)
P815B mTDO cl12	166.8	0.0	5,483	136			4,090 (±24)	128 (±8)

*Expression of human or mouse *TDO2* and *IDO1* mRNA was measured by quantitative RT-PCR, normalized to actin and expressed as the amount of mRNA molecules per cell. Each cDNA was tested at least in duplicate.

[†]Enzymatic activity was estimated by measuring tryptophan degradation and kynurenine production in the supernatant of 14-h (murine lines) or 24-h (human lines) cultures performed in the absence or presence of TDO inhibitor 680C91 (20 μ M), IDO1 inhibitor 1-methyl-L-tryptophan (1MT, 400 μ M) or both. Tryptophan and kynurenine were measured by HPLC. ND, not determined.

[‡]Enzymatic activity (\pm SEM) was estimated by measuring kynurenine production in the clarified cell extracts with L-tryptophan at 1 mM in the absence or presence of TDO inhibitor 680C91 (25 μ M). The rate of catalysis was calculated within the linear phase of *N*-formylkynurenine production. Results are the mean value of three independent experiments performed in duplicate and with two measures for each duplicate.

Similarly, low levels of TDO were observed in early *concepts*, where they might contribute to tolerance of the embryo by the maternal immune system (31–33).

Using a TDO inhibitor, we proved, in a preclinical model, the concept that TDO inhibition promotes tumoral immune rejection. It will be of interest to evaluate the role of TDO in additional tumor

models expressing various levels of TDO. The adverse effects of TDO inhibition appear limited. TDO is normally expressed at a high level in the liver and at low level in the brain (34). We did not observe obvious toxicity in mice treated with LM10 for 3 months. Moreover, mice genetically deficient for *TDO2* were recently obtained and found viable and healthy (35). The main observation

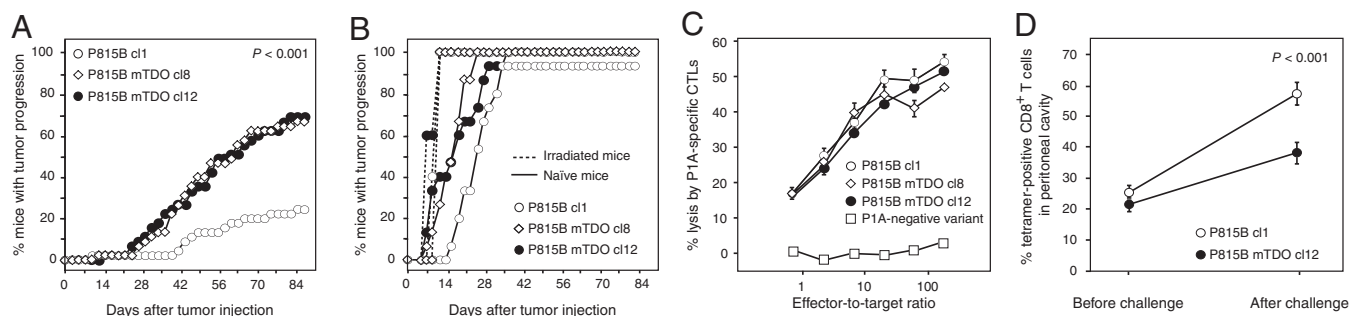
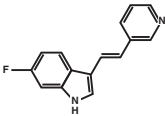
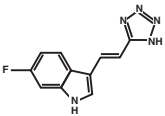


Fig. 1. Immune resistance of TDO-expressing tumors. (A) Mice ($n = 45$ per group) were immunized by i.p. injection of living L1210 cells expressing P1A and B7-1, and challenged 4 wk later by i.p. injection of 4×10^5 cells either P815B cl1 (\circ), or P815B-mTDO cl8 (\diamond), or P815B-mTDO cl12 (\bullet). Tumor progression was monitored. Shown are the compiled results of three experiments, each involving groups of 15 mice. $P < 0.001$ for cl1 versus cl8 and for cl1 versus cl12 (Logrank test). (B) Naive (solid line) ($n = 15$ per group) or irradiated mice (dashed line) ($n = 5$ per group, 650 cGy) were injected as in A. One representative experiment out of five is shown. (C) Lysis of TDO-transfected P815B cells by P1A-specific CTLs. P1A-specific CTLs were incubated with ⁵¹Cr-loaded TDO-transfected P815B cells at varying effector/target ratios. P15 variant P1.ista-B-, which has lost gene *P1A*, was used as a control target (\square) P1A-negative variant). One representative experiment out of three is shown. (D) Proportion of P1A-specific T cells among CD8⁺ T cells in the peritoneal cavity of immunized mice, estimated using H-2L^d/P1A tetramers 1 d before or 4 d after i.p. challenge with 10^6 cells of P815B cl1 (\circ , $n = 30$ mice) or P815B-mTDO cl12 (\bullet , $n = 30$ mice). Error bars represent SEM. $P < 0.001$ for cl1 versus cl12 after challenge (two-tailed Student *t* test). One representative experiment out of two is shown.

A

		680C91		LM10	
Structure					
		$\mu\text{g/ml}$	μM	$\mu\text{g/ml}$	μM
K_i hTDO		0.21	0.88	1.28	5.6
IC50 mTDO		0.35 ± 0.06	1.46 ± 0.26	0.45 ± 0.12	1.96 ± 0.54
IC50 mIDO		> 19	> 80	> 91.6	> 400
IC50 hTDO		0.07 ± 0.05	0.28 ± 0.22	0.14 ± 0.02	0.62 ± 0.11
LD50		> 19	> 80	> 91.6	> 400
Solubility	pH 1	> 71.4	> 300	52.4	229
	pH 7.4	21.9	92	> 68.7	> 300
	pH 9	18.6	78	> 68.7	> 300
Plasma concentration	Day 1	0.17 ± 0.05	0.72 ± 0.2	19.3 ± 4.3	84.5 ± 18.8
	Day 2	0.08 ± 0.05	0.34 ± 0.2	33.3 ± 7.3	145.3 ± 31.8
	Day 7	0.13 ± 0.05	0.55 ± 0.2	41.3 ± 4.7	180.2 ± 20.4

B

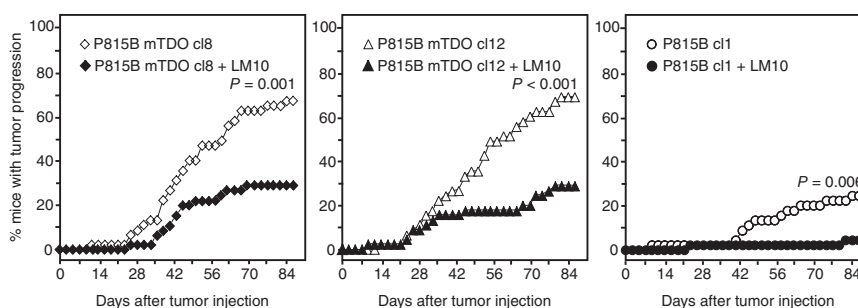


Fig. 2. Reversal of immune resistance by systemic inhibition of TDO. (A) Structure, activity, physicochemical features, and bioavailability of TDO inhibitors 680C91 and LM10. K_i were measured on recombinant human TDO (hTDO) (16). A cellular assay based on mouse P815B-mTDO, mouse P815B-mIDO, or human 293E-hTDO cells was used to measure IC50, defined as the concentration giving 50% inhibition of TDO activity at a L -tryptophan concentration of 80 μM . Values \pm SEM are shown corresponding to the mean of three independent experiments. LD50 was estimated by an MTT assay evaluating cell viability in the same cultures. Solubility was evaluated at room temperature as described in ref. 16. Bioavailability was estimated by measuring plasma concentration of the compounds after 1, 2, or 7 d of oral administration of 160 mg/kg/day. Mice ($n = 10$) received either normal drinking water or a solution of 1 mg/mL 680C91 at pH 2.5 or 1 mg/mL LM10 at pH 9, of which they drank an average of 4 mL/day. (B) Tumor progression in immunized mice ($n = 45$ per group) challenged by i.p. injection of 4×10^5 cells from either P815B-mTDO cl8 (diamonds, *Left*), P815B-mTDO cl12 (triangles, *Center*), or P815B cl1 (circles, *Right*) and treated (black symbol) or not (white symbol) with 160 mg/kg/day LM10 in the drinking water, starting one day before the injection of tumor cells. Shown are the compiled results of three experiments, each involving groups of 15 mice. The untreated groups are identical to those shown on Fig. 1A. Mice received either normal drinking water or a solution of LM10 at 1 mg/mL $P = 0.001$, $P < 0.001$, and $P = 0.006$ for treated versus untreated mice after challenge with cl8, cl12 and cl1, respectively (Logrank test).

in those mice is a reduced anxiety, which may result from their highly increased serum levels of tryptophan. Indeed, tryptophan is the precursor of serotonin, and TDO inhibitors were previously considered as potential antidepressants (10).

A recent report published during the reviewing process of this paper corroborates our findings by showing that TDO is ex-

pressed constitutively in human glioblastomas and promotes tumor progression through the production of kynurenine acting as endogenous ligand of the aryl hydrocarbon receptor, resulting in increased tumor cell survival and motility, and reduced anti-tumor immune responses (36). In our tumor model, however, we did not observe a tumor cell autonomous effect of TDO activity,

Table 3. Evaluation of liver toxicity of LM10 in mice

Liver enzyme*	ALAT (IU/mL \pm SEM)	ALAP (IU/mL \pm SEM)	ASAT (IU/mL \pm SEM)	γ -GT (IU/mL \pm SEM)
Control [†]	34.8 ± 2.4	74.4 ± 2.3	106.7 ± 8.8	3.0 ± 0.2
LM10-treated [‡]	47.5 ± 3.3	92.7 ± 3.0	156.9 ± 8.1	2.8 ± 0.2
Normal range [§]	21–83	68–89	70–215	1–4

*Enzyme activities were measured in the plasma. ALAP: alkaline phosphatase; γ -GT: γ -glutamyl transferase; ALAT: alanine aminotransferase, ASAT: aspartate aminotransferase.

[†]Group of 43 mice from the experiment reported in Fig. 2B, fed with normal drinking water and tested 107 d after tumor challenge. Shown are the compiled results of three independent assays.

[‡]Group of 82 mice from the experiment reported in Fig. 2B, fed with a solution of LM10 (1 mg/mL pH 9; 160 mg/kg/day) and tested 107 d after tumor challenge. Shown are the compiled results of three independent assays.

[§]Group of 7 untreated normal DBA/2 mice.

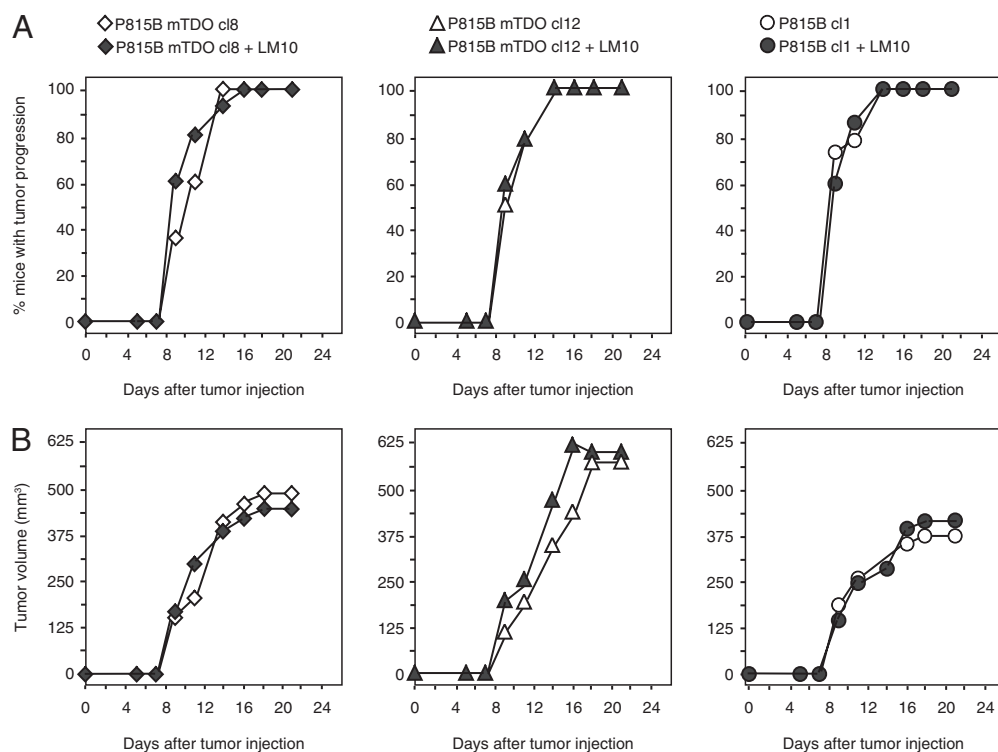


Fig. 3. Lack of antitumor effect of LM10 in RAG2 knockout mice. RAG2 knockout mice (15 per group) received a s.c. injection of 2×10^5 cells of the indicated P815 tumor clones (either P815B-mTDO cl8 (diamonds, *Left*), P815B-mTDO cl12 (triangles, *Center*), or P815B cl1 (circles, *Right*), and were treated (filled symbols) or not (open symbols) with 160 mg/kg/day LM10 in the drinking water (1 mg/mL) as in Fig. 2. The proportion of mice bearing progressive tumors in *A* and the mean tumor volume in *B* are reported. Tumor volume was calculated with formula $(\pi \times L \times l^2)/12$. One representative experiment is shown out of two.

as TDO inhibition *in vivo* resulted in tumor control in immune competent but not in immunodeficient mice (Fig. 3).

Table 4. *TDO2* and *IDO1* expression in human tumor cell lines

Tumor type	<i>TDO2</i> only	<i>IDO1</i> only	<i>TDO2</i> and <i>IDO1</i>	Total
Colorectal carcinoma	5/11	1/11	0/11	6/11
Glioblastoma	1/8	3/8	1/8	5/8
Leukemia	1/4	0/4	0/4	1/4
Lymphoma	0/4	0/4	0/4	0/4
Melanoma	0/12	2/12	0/12	2/12
Mesothelioma	1/7	2/7	3/7	6/7
Myeloma	1/3	0/3	0/3	1/3
Head and neck carcinoma	1/11	2/11	5/11	8/11
Ovarian carcinoma	0/1	1/1	0/1	1/1
Pancreatic adenocarcinoma	0/5	3/5	1/5	4/5
NSCLC	5/7	0/7	1/7	6/7
SCLC	0/6	0/6	0/6	0/6
Sarcoma	0/6	0/6	2/6	2/6
Breast carcinoma	1/4	1/4	0/4	2/4
Bladder carcinoma	0/3	1/3	2/3	3/3
Hepatocarcinoma	0/2	0/2	0/2	0/2
Gall-bladder carcinoma	0/1	0/1	1/1	1/1
Renal cell carcinoma	4/9	1/9	0/9	5/9
Total	20/104	17/104	16/104	53/104

Expression of *TDO2* and *IDO1* was evaluated by RT-PCR on RNA isolated from human tumor cell lines. NSCLC: non small cell lung carcinoma, SCLC: small cell lung carcinoma. In some tumor types, such as melanoma, the proportion of *TDO2*-expressing tumor lines is lower than the proportion of *TDO2*-expressing tumor samples (see Table 1). Two factors may explain this difference: (i) during establishment of the cell lines, TDO-expressing tumor cells may have been counterselected because of their tryptophan-depleting effect, as in most cases the cell lines were established in the absence of TDO inhibitor, and (ii) TDO might also be expressed by some stromal cells, such as activated macrophages, which are present in tumor samples and absent in tumor cell lines.

Blocking both TDO and IDO1 to improve the efficacy of cancer immunotherapy would be complementary, not redundant: in a series of 104 human tumor lines of various histological types, we observed 20 tumors expressing only *TDO2*, 17 expressing only *IDO1* and 16 expressing both (Table 4). Therefore, targeting both IDO1 and TDO would allow reaching 51% of tumors instead of 32% with IDO1 or 35% with TDO alone. Moreover, our results suggest that TDO inhibition might be useful also for tumors that do not intrinsically express TDO. If confirmed, this notion would further increase the proportion of tumors eligible for such a therapy.

In conclusion, pharmacological inhibition of TDO might represent a safe and efficient approach for cancer therapy acting by promoting tumoral immune rejection, thereby leveraging cancer immunotherapy.

Materials and Methods

Mice. DBA/2 mice aged 6–8 wk were purchased from Harlan, provided food and water *ad libitum*, and housed under specific pathogen-free conditions. Animals were immunized by injection of 10^6 live L1210.P1A.B7-1 cells into the peritoneal cavity and challenged 4 wk later by i.p. injection of 4×10^5 tumor cells, as described (4, 12). As indicated, mice were given LM10 in the drinking water (1 mg/mL, pH 9), of which they drank an average of 4 mL/day. For assessment of bioavailability, naïve mice ($n = 10$) were given a solution of 680C91 (1 mg/mL, pH 2.5) or LM10 (1 mg/mL, pH 9) instead of drinking water. Blood was collected from the retro-orbital sinus in Lithium-Heparin tubes (Microvette 500 LH, Sarstedt). After centrifugation at $11,000 \times g$ for 10 min, plasma was collected and the concentration of compound was measured by HPLC-UV at 345 nm (680C91) or 326 nm (LM10). For the HPLC analysis, 50 μ l of supernatant were mixed with 500 μ l of acetonitrile to precipitate the proteins. After centrifugation, the supernatant was collected, concentrated in a speedvac, resuspended in a final volume of 100 μ l of water, and injected onto an Onyx Monolithic C18 column (Phenomenex). Hepatic enzymes were assayed on a Synchron CX system according to the manufacturer's instructions (Beckman Coulter). The ethical committee of the Faculty of Medicine, Université Catholique de Louvain, approved all mouse experiments.

Further Details. See *SI Materials and Methods* for details about:

- Cell lines and tumor samples
- Cellular assay for TDO and IDO activity
- Enzymatic assay for TDO activity on crude extracts

- Lysis assay
- Tetramer staining and fluorescence-activated cell sorting analysis
- Quantitative RT-PCR analysis of mRNA expression
- Cellular assay for TDO inhibition
- Compounds synthesis and characterization
- Synthesis of *trans*-3-(6-fluoro-1*H*-indol-3-yl)acrylonitrile
- Synthesis of *trans*-6-Fluoro-3-[2-(1*H*-tetrazol-5-yl)vinyl]-1*H*-indole (LM10)
- Statistics.

ACKNOWLEDGMENTS. We are grateful to Dr. Lionel Pochet and Laurence Moineaux for their assistance in solubility evaluation and chemical syn-

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