

Hydroxyamidine Inhibitors of Indoleamine-2,3-dioxygenase Potently Suppress Systemic Tryptophan Catabolism and the Growth of IDO-Expressing Tumors

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Abstract

Malignant tumors arise, in part, because the immune system does not adequately recognize and destroy them. Expression of indoleamine-2,3-dioxygenase (IDO; IDO1), a rate-limiting enzyme in the catabolism of tryptophan into kynurenine, contributes to this immune evasion. Here we describe the effects of systemic IDO inhibition using orally active hydroxyamidine small molecule inhibitors. A single dose of INCB023843 or INCB024360 results in efficient and durable suppression of IDO1 activity in the plasma of treated mice and dogs, the former to levels seen in *Ido1*-deficient mice. Hydroxyamidines potently suppress tryptophan metabolism *in vitro* in CT26 colon carcinoma and PAN02 pancreatic carcinoma cells and *in vivo* in tumors and their draining lymph nodes. Repeated administration of these IDO1 inhibitors impedes tumor growth in a dose- and lymphocyte-dependent fashion and is well tolerated in efficacy and preclinical toxicology studies. Substantiating the fundamental role of tumor cell-derived IDO expression, hydroxyamidines control the growth of IDO-expressing tumors in *Ido1*-deficient mice. These activities can be attributed, at least partially, to the increased immunoreactivity of lymphocytes found in tumors and their draining lymph nodes and to the reduction in tumor-associated regulatory T cells. INCB024360, a potent IDO1 inhibitor with desirable pharmaceutical properties, is poised to start clinical trials in cancer patients. *Mol Cancer Ther*; 9(2); 489–98. ©2010 AACR.

Introduction

With the encouraging results from large-scale clinical trials of immune system–provoking therapies (1–3), interest has again begun to swell around the promise of harnessing the power of the immune system to attack cancers. Approaches focused on stimulating the immune system to recognize and destroy tumors have included monoclonal antibodies, autologous cell vaccines, and cytokine therapies (reviewed in ref. 4). Early in their development, these strategies worked in only a small percentage of patients, with objective responses in most studies around 10% (5, 6). Although progress has been made in identifying markers that will prospectively select those patients who will benefit most (7), it is desirable to understand the reasons these therapies are not more broadly active and to develop additional immune modulating agents. Suppressing mechanisms in place to rein in

normal immune responses may be usurped by tumor cells to limit the effectiveness of antitumor responses (8). Interference with two of these, CTLA4 and the PD1/PDL1 axis, is currently being tested clinically and providing a similar low-frequency but durable benefit to patients (2, 3, 9). Another such mechanism under investigation is tryptophan depletion through enhanced indoleamine-2,3-dioxygenase (IDO) activity.

The proliferation and function of lymphocytes are exquisitely sensitive to decreases in tryptophan levels and increases in proapoptotic metabolites of tryptophan catabolism (10). Two related enzymes process tryptophan into kynurenine and its downstream catabolites: IDO (IDO1), expressed by cells of the immune system in an inducible manner, and tryptophan 2,3-dioxygenase (TDO), expressed in the liver and responsible for catabolizing dietary tryptophan. The role of an additional tryptophan-catabolizing enzyme, IDO2, is uncertain at this point, as there are functionally inactive polymorphisms found in approximately 50% of examined populations with limited or no correlation to tumorigenesis (11, 12). However, based on the well-known role of IDO in fetal immune privilege, it is clear that IDO is important for the function of the immune system. The expression of IDO by activated dendritic cells can serve to activate regulatory T cells (Tregs), thereby constituting one mechanism by which the immune system can restrict excessive

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lymphocyte reactivity (13). This mechanism has also been adopted by tumor cells to disable the immune system, permitting tumor progression. The pronounced expression of IDO by tumor cells has been documented in several tumor types (14) and has been correlated with poor prognosis in patients with colon and ovarian cancers and melanomas (15–17), among others. It has been shown in several studies that sera from cancer patients have higher kynurenine/tryptophan ratios than sera from normal volunteers, consistent with increased IDO activity (16, 18, 19). In preclinical models, *Ido1* expression by immunogenic tumors prevents rejection (14), silencing of IDO expression in tumor cells enhances tumor-specific killing (20), and the incidence and growth of 7,12-dimethylbenz(a)anthracene-induced premalignant skin papillomas is decreased in *Ido1*^{-/-} mice (21). Taken together, these data suggest that modulating kynurenine generation through IDO inhibition might prove beneficial to cancer patients.

Ideally, a clinical inhibitor of IDO1 activity would potentially inhibit tryptophan metabolism locally – in the tumor and associated microenvironment – as well as in the draining lymph nodes, where tumor antigen presentation may be most effective. Moreover, to minimize undesirable side effects, inhibitors should be inactive against the related IDO enzyme. Here, using *Ido*-expressing tumor models, we characterize the activity of two such orally active agents, INCB023843 and INCB024360. These hydroxyamidines inhibit the conversion of tryptophan to kynurenine in tumor cell lines. *In vivo*, they decrease plasma kynurenine concentrations in wild-type mice to those seen in *Ido1*-deficient mice, suggesting these compounds can completely block IDO1 function. Importantly, these compounds were efficacious in multiple tumor models where they potentially affected kynurenine generation in tumors and their associated draining lymph nodes, resulting in tumor growth control (TGC; see Materials and Methods for details) through a lymphocyte-dependent mechanism.

Materials and Methods

Cell Lines and Compounds

CT26 murine colon carcinoma cells (obtained from the American Type Culture Collection) were maintained in DMEM supplemented with 10% fetal bovine serum (FBS). PAN02 murine pancreatic ductal adenocarcinoma cells (obtained from the Division of Cancer Treatment and Diagnosis, NCI, NIH Tumor Repository) were maintained in RPMI 1640 supplemented with 10% FBS. INCB023843 and INCB024360 were synthesized at Incyte as described previously (22, 23).

Cell Based Determinations of Tryptophan and Kynurenine

Both CT26 and PAN02 cells were plated at 3×10^5 cells/well in 6-well plates (Corning), and allowed to adhere overnight. The next day, the media were replaced,

and appropriate wells received recombinant murine IFN γ (R&D Systems) to a final concentration of 100 ng/mL, whereas an unstimulated control received diluent. At that time, cells received IDO inhibitors across a 10-point dilution scheme. After 48 h, media were collected and centrifuged to remove dead cells and stored at -20°C until liquid chromatography/mass spectrometry (LC/MS) analysis as described below for tissue and plasma samples in the Pharmacodynamic Analyses section.

Animal Studies

Animals were housed in barrier facilities fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International. All of the procedures were conducted in accordance with the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals and with Incyte Animal Care and Use Committee guidelines in the case of mouse studies or with Charles River Laboratories Animal Care and Use Committee guidelines in the case of beagle studies.

Pharmacokinetic-Pharmacodynamic Studies

To determine the effect of IDO inhibition on plasma kynurenine, fed C57BL/6 wild-type (The Jackson Laboratory, Fig. 1A; or Charles River, Fig. 1B) or *Ido1*^{-/-}-deficient mice (B6.129-*Ido1*^{tm1A1m}/J, The Jackson Laboratory) were administered a single oral dose of INCB023843 or INCB024360, at which point food was removed from the cages until after the 8-h time point. At various time points after dosing, mice were euthanized and blood was collected by cardiac puncture. To determine the effect of IDO inhibition on plasma kynurenine in a nonrodent species, fed male beagle dogs were administered a single dose of INCB023843, at which point food was removed from the cages until after the 12-h time point. Blood was collected at various time points after dosing. Plasma was analyzed for the presence of INCB023843, INCB024360, tryptophan, and kynurenine according to the methods below. Data were analyzed using one-way ANOVA with Dunnett's posttest for statistical significance.

Syngeneic Tumor Models

For the CT26 model, 8-week-old female Balb/c or Balb/c *nu/nu* mice (Charles River) were inoculated s.c. with 1×10^6 tumor cells. For the PAN02 model, 8-week-old female C57BL/6 mice, Balb/c *nu/nu* (Charles River), or *Ido1*-deficient (*Ido1*^{-/-}) mice were inoculated s.c. with 3 to 5×10^6 tumor cells. Tumor sizes were measured after becoming visible two or three times weekly in two dimensions using a caliper, and the volume presented in mm^3 using the formula: $V = 0.5(A \times B^2)$, where *A* and *B* are the long and short diameters of the tumor, respectively. Tumor-bearing animals were sorted into groups with similar mean tumor volumes prior to treatment, usually 100 to 200 mm^3 . Treatments are listed in each experiment. Each day of the oral dosing studies, free base INCB023843 and INCB024360 were reconstituted in 3% N,N-Dimethylacetamide, 10% (2-Hydroxypropyl) β -Cyclodextrin. For studies

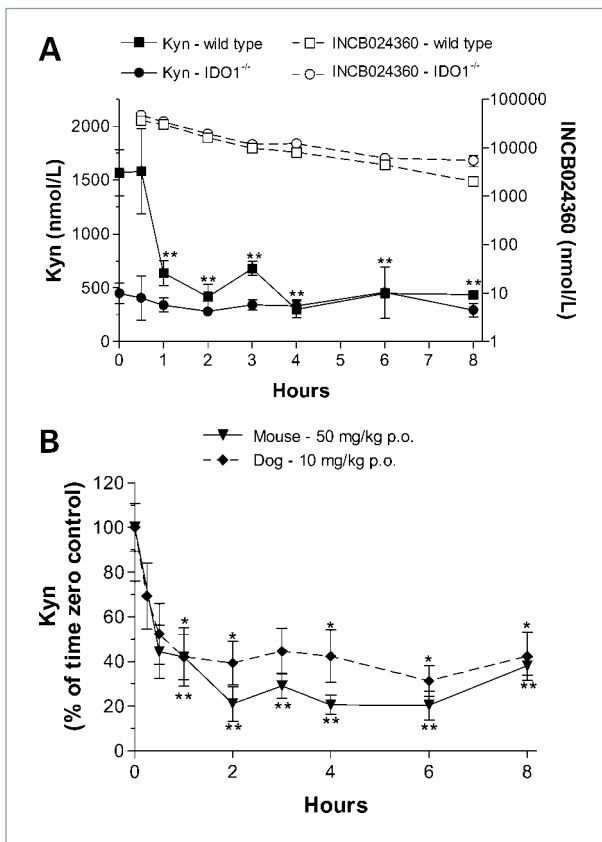


Figure 1. Hydroxyamidines suppress kynurenine (Kyn) efficiently in multiple species. A, INCB024360 reduces kynurenine levels in wild-type mice to levels seen in *Ido1*^{-/-} mice. Wild-type (■) and *Ido1*^{-/-} (●) female C57BL/6 mice were dosed orally with 50 mg/kg INCB024360 and blood was harvested at the indicated times. Average values from the plasma for three mice (±SE), analyzed for kynurenine (solid symbols, left axis) and INCB024360 levels (open symbols, right axis) by LC/MS/MS, are shown. All data between 1 and 8 h for effects in wild-type mice are statistically significant (**, $P < 0.01$). B, INCB023843 reduces kynurenine levels similarly in mice and dogs. INCB023843 was administered orally to female C57BL/6 mice (▼, 50 mg/kg) and male beagle dogs (◆, 10 mg/kg). Blood was harvested at the indicated times. Average values from the plasma for three animals (±SE), analyzed for kynurenine by LC/MS/MS, are shown. All data between 1 and 8 h are statistically significant [$P < 0.01$ (**) for mice and $P < 0.05$ (*) for dogs, except at the 3-h time point].

with s.c. pumps, INCB023843 and INCB024360 were reconstituted in 40% *N,N*-Dimethylacetamide, 60% propylene glycol. Body weights were monitored throughout the study as a gross measure of toxicity/morbidity. TGC, expressed in %, is calculated using the formula: $1 - [(treated \text{ (day X)} - treated \text{ (day Y)}) / (vehicle \text{ (day X)} - vehicle \text{ (day Y)})]$, where X is the day of last or interim measurement and Y is the day dosing commenced. Data were analyzed using one-way ANOVA with Dunnett's posttest for statistical significance. Plasma concentration of INCB024360, tryptophan, and kynurenine were determined by LC/MS/MS analysis following retro-orbital or cardiac puncture blood collection. In certain experiments, tumors and tumor-draining lymph nodes (TDLN) were also harvested

for the determination of INCB023843, INCB024360, tryptophan, and kynurenine.

Determination of Tryptophan, Kynurenine, and INCB024360 by Analytical Methods

Pharmacodynamic Analyses. An analytical method for the quantification of tryptophan, kynurenine, INCB023843, and INCB024360 was developed. The method combined a protein-precipitation extraction using trichloroacetic acid and LC/MS/MS analysis. It showed a linear assay range from 20 to 10,000 nmol/L for kynurenine, INCB023843, and INCB024360, and 200 to 100,000 nmol/L for tryptophan, analyzing 0.1 mL samples. Plasma samples were diluted 10-fold in water. Tissues were homogenized in 5% acetonitrile in water with 0.1% formic acid. The tissue dilution depended upon the mass of tissue (i.e., weight to volume ratio of 3 for tumors and 10 for lymph nodes). The homogenates were spun to allow for sampling of the supernatant. Aqueous standards were prepared to alleviate the need for adjustment for endogenous tryptophan and kynurenine present in biological matrices.

Pharmacokinetic Analyses. An analytical method for the quantification of INCB023843 and INCB024360 was developed. The method combined a protein-precipitation extraction using acetonitrile and LC/MS/MS analysis. It showed a linear assay range from 2 to 10,000 nmol/L, analyzing 0.1 mL samples.

Preparation, Stimulation, and Analysis of Lymphocyte Function

Tumor-infiltrating lymphocytes (TIL) were harvested from tumors after treatment *in vivo*. Using sterile scissors and forceps, tumors were dissociated in 6 to 7 mL cold PBS/2% FBS and then crushed with the flat base of the plunger of a 10-cc syringe. This preparation was transferred to a prewetted nylon mesh (BD Falcon) in a sterile 50-mL conical tube. Cells were washed and adjusted to 10^7 cells/mL. This suspension was subjected to mouse PBMC isolation medium (Cedarlane) and centrifuged for 20 min at 2,500 rpm. Cells in the interface (herein considered TILs) were carefully harvested and transferred to a sterile tube, where they were washed twice with cold PBS/2% FBS. TILs were normalized for CD3-positive cells by flow cytometry and were cultured alone in RPMI, 5% heat inactivated FBS, 1 mmol/L HEPES, nonessential amino acids, 25 μ mol/L 2-mercaptoethanol, sodium pyruvate, and penicillin/streptomycin. Lymphocytes were isolated from the TDLNs of treated mice by physical disruption of the lymph nodes in cold PBS using a Wheaton tissue grinder (Fisher). Lymph node cells were washed twice prior to plating in the presence of 50 ng/mL phorbol 12-myristate 13-acetate (Sigma-Aldrich) and 500 ng/mL ionomycin (Sigma-Aldrich) for 24 h. After 24 h, cell culture supernatants from unstimulated TIL cultures and stimulated lymph node cultures were harvested for the determination of IFN γ levels using the mouse IFN γ ELISA kit (R&D Systems). Two-color staining of lymphocytes was

done with Fluorescein isothiocyanate- and phycoerythrin-conjugated anti-CD3, -CD4, -CD8, and -CD25 (BD/Pharmingen). Fluorescence-activated cell sorting (FACS) analysis of 20,000 to 100,000 gated lymphocytes per sample was done with a FacsCalibur flow cytometer running CellQuest Software (both Becton Dickinson).

Results

Hydroxyamidines Effectively Suppress Tryptophan Catabolism *In vivo* in Multiple Species

Because IDO is the rate-limiting enzyme that converts tryptophan to kynurenine, its activity can be detected in both cells and tissues as an increase in kynurenine levels. We have previously shown that hydroxyamidine compounds can suppress IDO activity *in vitro* and when administered by s.c. injection (23); however, none of those compounds had pharmaceutical properties suitable for sustained IDO inhibition. We utilized hydroxyamidines with optimized pharmaceutical properties, such as moderate plasma protein binding and suitable cell permeability consistent with oral dosing, to suppress kynurenine levels *in vivo* (22, 23). In naïve C57BL/6 mice, 50 mg/kg INCB024360 decreased plasma kynurenine levels within 1 hour and those levels stayed at least 50% suppressed through the 8-hour time course (Fig. 1A; $P < 0.01$). To confirm that the decreased kynurenine levels observed in wild-type mice resulted specifically from IDO1 inhibition, *Ido1*^{-/-} mice were dosed as above. Consistent with specific inhibition of *Ido1* no reduction in kynurenine levels was observed in the *Ido1*^{-/-} mice where kynurenine (approximately 20-25% of kynurenine in wild-type mice) was generated by other tryptophan-catabolizing enzymes (e.g., *Tdo* or *Ido2*; Fig. 1A). This was seen despite similar compound exposures between mouse strains (Fig. 1A). Further, during maximal suppression of *Ido* by INCB024360 in wild-type mice, the plasma kynurenine levels were quite similar to those present at baseline in the *Ido1*^{-/-} mice, suggesting >90% inhibition of *Ido1* activity by INCB024360.

To ensure that these observations were not compound specific, we dosed mice with a structurally related hydroxyamidine INCB023843 (22, 23) and found similar results (Fig. 1B). Additionally, we wanted to examine whether this compound could suppress kynurenine levels in a nonrodent species. Male beagle dogs were administered a single oral dose of 10 mg/kg INCB023843 and the activity seen in dogs was similar to that in mice (Fig. 1B; $P < 0.05$). Curiously, Boasso et al. were unable to detect an effect on kynurenine using repeat dosing of D-1MT, a described IDO2 inhibitor currently in clinical trials, in treated macaques (24). In aggregate, these data represent (to the best of our knowledge) the first demonstrable systemic inhibition of IDO activity using an oral small molecule IDO1 inhibitor in higher species.

Hydroxyamidines Suppress Kynurenine Generation in IDO-Expressing Murine Tumor Cells

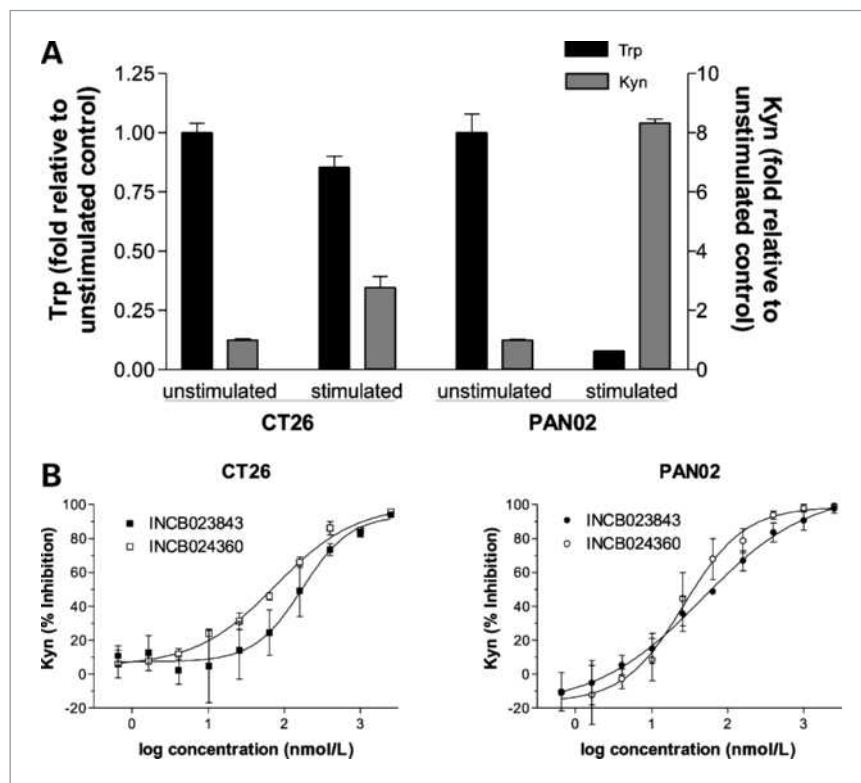
Although we had previously shown that hydroxyamidines could suppress IDO activity in human HeLa cells, these cells could not be used to develop an *in vivo* model for testing the efficacy of IDO inhibitors in immune competent animals. Knowing that a large percentage of human tumors express IDO1 but that many mouse cells lines do not, even upon IFN γ stimulation (25), we sought to identify multiple murine tumor models where IDO could be expressed. Human colon and pancreatic carcinomas have been shown to express IDO1 at high levels by immunohistochemistry (14), and as such, focus was placed here. We observed that treatment of CT26 murine colon carcinoma cells with 100 ng/mL IFN γ induced tryptophan catabolism with a modest decrease in tryptophan levels and a 3-fold increase in kynurenine levels (Fig. 2A). PAN02 pancreatic carcinoma cells had enhanced responses to IFN γ *in vitro*, with an 8-fold increase in kynurenine generation paired with a near-complete depletion of tryptophan in the media. INCB023843 and INCB024360 restored tryptophan levels to those seen in DMSO-treated controls and significantly impaired kynurenine generation in both cell lines with IC₅₀ values of 172 and 76 nmol/L, respectively, for CT26 cells and 46 and 27 nmol/L, respectively, for PAN02 cells (Fig. 2B). Hydroxyamidines seem to be slightly less potent on cells expressing murine *Ido* than those expressing human *Ido*. For example, there was a >4-fold shift in potency between HEK293 cells transfected with human (15 nmol/L) and mouse *Ido1* (66 nmol/L) for INCB024360.¹ Importantly, neither cell line was sensitive to growth inhibition by these compounds. Because this allows us to study immune-mediated effects more readily, these compounds were selected for further testing *in vivo*.

Systemic IDO Inhibition Impedes Tumor Growth in the Absence of Toxicity

Although IDO was inducible by IFN γ in cultured cells, we processed tumor fragments and measured IDO expression by quantitative PCR to confirm IDO expression by tumors. Both CT26 and PAN02 tumors expressed *Ido1* to high levels (191-fold and 35-fold increased over cultured cells, respectively) and therefore could be used for determining the effects of IDO1 inhibition on tumor growth. Balb/c mice bearing well-established CT26 colon carcinomas were implanted with s.c. pumps delivering 50 mg/kg/d (based on a mouse starting weight of 20 g) INCB023843 or INCB024360 or vehicle. Both agents inhibited CT26 tumor growth, with 57% and 54% TGC for INCB023843 and INCB024360, respectively ($P < 0.05$; day 25, Fig. 3A). Because INCB023843 and INCB024360 performed equivalently in multiple *in vitro* and *in vivo* assessments, the two compounds were used interchangeably in subsequent studies.

¹ P.A. Scherle and G. Yang unpublished results.

Figure 2. IFN γ induces IDO activity in murine tumor cell lines, the activity of which is completely inhibited by INCB023843 and INCB024360. A, after a 48-h stimulation with 100 ng/mL IFN γ , subconfluent cultures of CT26 and PAN02 cells exhibit a decrease in media tryptophan levels (black bars, left axis) and a concomitant accumulation of kynurenine (grey bars, right axis). B, this induced activity is completely inhibited by concurrent treatment with IDO inhibitors, INCB023843 and INCB024360. Both experiments, mean values \pm SD of at least two experiments. Cell supernatants were analyzed by LC/MS/MS for tryptophan and kynurenine.



To investigate the potential for INCB024360 as an oral agent, we administered increasing doses to CT26 tumor-bearing mice. There was a dose-dependent inhibition of tumor growth with 34% and 57% TGC seen with 30 ($P < 0.05$) and 100 mg/kg ($P < 0.01$) bid, respectively (day 21, Fig. 3B). These data were similar to those obtained with INCB023843, where 36% and 54% TGC was seen with 30 and 100 mg/kg bid, respectively. Dose- and time-dependent decreases in kynurenine levels were seen in plasma samples from these tumor-bearing mice. In this experiment, $>50\%$ suppression was seen for at least 16 hours with the 30 mg/kg dose and for approximately 24 hours with the 100 mg/kg dose over the course of the day (Table 1, day 1 of dosing), which was expected because the levels of circulating inhibitor exceeded the IC_{50} values for both tumor cell lines (Fig. 2B), even when accounting for plasma protein binding (approximately 4% free). Given the proposed mechanism of action that IDO inhibitors would require the activity of lymphocytes, neither agent inhibited the growth of CT26 tumors in Balb/c *nu/nu* mice, which lack an intact immune system (data not shown). Importantly, *Ido1* is still expressed by CT26 tumors grown in Balb/c *nu/nu* mice. These data imply that hydroxyamidines can, in the presence of a functional immune system, impede tumor growth as single agents and that continuous inhibition ($>50\%$) of *Ido1* provides better tumor growth control than periodic inhibition.

Importantly, systemic *Ido* inhibition proved to be well tolerated in both efficacy and formal 28-day toxicology

studies in which doses of up to 2,000 mg/kg/d and 500 mg/kg/d were administered to mice and dogs, respectively. No adverse findings in any parameter (clinical observations, body weight, food consumption, clinical pathology, ophthalmology, and histopathology) were identified, nor were there signs of autoimmunity (not shown). These studies suggest sizeable therapeutic margins (>30 -fold) over projected compound exposure in humans at doses that effectively inhibit IDO.

Inhibition of Kynurenine Generation in Tumors and Lymph Nodes

Having shown that hydroxyamidines can suppress kynurenine levels in plasma, we next determined the extent of *Ido1* inhibition in the desired target tissues, namely, tumors and draining lymph nodes. CT26 tumor-bearing Balb/c mice were treated with 100 mg/kg INCB024360 orally twice daily for 12 days and tissues were harvested 2 hours after the last dose (Fig. 4). Detection of much higher levels of kynurenine in tumor tissue (~ 20 -fold) when compared with plasma and lymph nodes suggested that the increased expression of *Ido1* by this tumor resulted in functional enzymatic activity. Importantly, kynurenine levels were similarly suppressed in all three tissues, ranging from 78% to 87%, compared with vehicle controls. INCB023843 treatment of CT26 tumors from a parallel study did not impact *Ido1* expression over 14 days, showing that the decreased kynurenine levels are not due to decreased *Ido1* expression (data not shown). These data

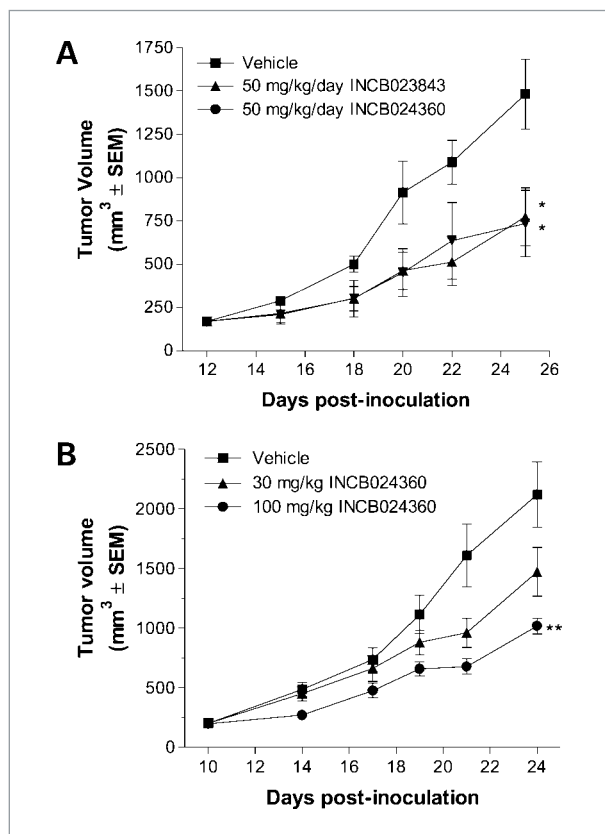


Figure 3. Hydroxyamidines are potent inhibitors of tumor growth. A, INCB023843 and INCB024360 inhibit CT26 colon carcinoma growth in Balb/c mice. Female Balb/c mice bearing CT26 tumors were given s.c. pumps delivering 50 mg/kg/d INCB023843 (▼), INCB024360 (▲), or vehicle (■). Mean tumor volumes (mm^3) \pm SE ($n = 8$ mice/group) are shown from the initiation of dosing ($\sim 170 \text{ mm}^3$). Both groups are statistically significant (*, $P < 0.05$) on day 25. B, INCB024360 inhibits CT26 colon carcinoma growth in a dose-dependent fashion. Female Balb/c mice bearing CT26 tumors were treated with 30 (▲) or 100 (●) mg/kg INCB024360 orally twice daily bid or vehicle (■). Mean tumor volumes (mm^3) \pm SE ($n = 8$ mice/group) are shown from the initiation of dosing ($\sim 200 \text{ mm}^3$). Data are from one of three experiments with similar results. Both groups are significantly significant (*, $P < 0.05$ for 30 mg/kg; **, $P < 0.01$ for 100 mg/kg) on day 21.

suggest that tracking kynurenine levels in plasma is sufficient for assessing systemic and hence tumor-associated IDO activity, at least with compounds possessing similar pharmaceutical characteristics. Therefore, reductions in plasma kynurenine levels may serve as a valuable pharmacodynamic marker in clinical studies.

Host IDO1 Expression Is Dispensable for Tumor Growth Suppression

As mentioned above, human pancreatic cancers also frequently express high levels of IDO. We have determined that PAN02 murine pancreatic cancer cells express inducible *Ido1* *in vitro* and express *Ido1* when grown as tumors (data not shown). Because these cells are syngeneic to the C57BL/6 background, PAN02 tumors can be

grown in wild-type, immunodeficient, and *Ido1*^{-/-} mice to determine the relative importance of *Ido1* expressed by host cells and *Ido1* expressed by tumors. In a manner similar to CT26 tumors in Balb/c mice, PAN02 tumors respond to INCB023843 (Fig. 5A) and INCB024360 (data not shown) in a dose-dependent fashion when grown in wild-type C57BL/6 mice. Consistent with the need for immune cells for tumor growth control, INCB023843 has no impact on PAN02 tumors grown in immunodeficient mice (Fig. 5B). Interestingly, INCB023843 inhibits the growth of PAN02 tumors in *Ido1*^{-/-} C57BL/6 mice, confirming the importance of tumor-derived IDO in tumor immune evasion, at least in this representative model system (Fig. 5C). Our studies have shown that *Ido1* expression by B16 tumors is negligible and this may account for the differences between our data with *Ido1*-expressing tumors and those shown with brassinins (a different class of IDO inhibitors) in B16F10 tumors (26). Our data agree with those described by Uyttenhove et al. (14) suggesting that IDO1 expression by the tumor is sufficient to block effective antitumor immune responses in otherwise immunocompetent hosts.

IDO Inhibition Increases the Number and Activity of Tumor Infiltrating Lymphocytes

The data above indicate that neutralizing IDO activity can control tumor growth and that this is dependent upon an intact immune system. Given that IDO1 inhibition is expected to work by impacting the activity of lymphocytes, we did a series of experiments studying the lymphocytes infiltrating CT26 and PAN02 tumors (TILs) and on lymphocytes from the TDLNs. One measure of lymphocyte function is the secretion of IFN γ . TILs were harvested from CT26 tumors after 7 days of INCB024360 treatment. There was enhanced basal activity of these cells, with a 6-fold increase in the specific release of IFN γ (Fig. 6A). Stimulation of lymphocytes from the TDLNs with phorbol myristate acetate and ionomycin showed that INCB024360 also enhances the responsiveness of lymphocytes, with a 50% increase in IFN γ secretion (Fig. 6B). This is similar to data obtained from lymphocytes from PAN02 tumor-bearing mice treated with INCB023843, where there was a 30% increase in IFN γ secretion (data not shown). To dissect which cell types are important to the control of tumor growth and the

Table 1. INCB024360 inhibits kynurenine generation in plasma in a dose-dependent fashion

	30 mg/kg INCB024360			100 mg/kg INCB024360		
	2 h	8 h	16 h	2 h	8 h	16 h
INCB024360 (nM)	4155	1151	228	23025	11946	1720
Kyn (% inhibition)	55%	68%	18%	75%	77%	47%

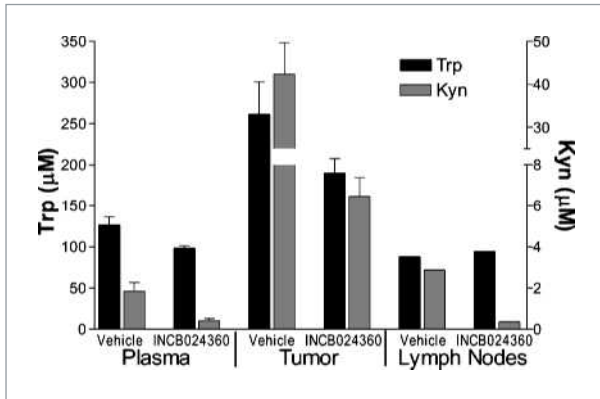


Figure 4. INCB024360 suppresses kynurenine equivalently in plasma, tumors, and lymph nodes. Female Balb/c mice bearing CT26 tumors were treated orally twice daily for 12 d with vehicle or INCB024360 at 100 mg/kg. Plasma, tumors, and TDLNs were harvested and tryptophan (black, left axis) and kynurenine (gray, right axis) levels were determined by LC/MS/MS.

mechanism of enhanced reactivity, the prevalence of various T cell subsets within tumors was analyzed. PAN02 tumor-bearing C57BL/6 mice were treated with INCB023843 for 7 days and TILs were harvested. As seen in Table 2, INCB023843 increased the percentage of CD3⁺, CD8⁺ and CD4⁺ T cells within PAN02 tumors, ranging from 1.7- to 2.1-fold. Despite the increase in infiltrating CD4⁺ cells, INCB023843 specifically decreased the percentage of CD4⁺CD25⁺ Tregs to approximately 30% of that found in the control mice. Thus, a shift in the ratio of effector to regulatory T cells (Teff/Treg) from 0.5 to a favorable 3.5 may explain the enhanced reactivity of lymphocytes in the tumors. These results are consistent with a role for IDO in activating Tregs.

Discussion

Although most immunomodulatory agents being tested clinically attempt to positively engage the immune system to attack tumor cells, a growing understanding of the suppressive mechanisms underlying the restriction of immune hyperreactivity highlights the need to interfere with these pathways as well. Interference with IDO, a tryptophan-catabolizing enzyme expressed by a wide range of human tumors, represents one such opportunity.

Here we characterized the effects of potent and selective orally bioavailable hydroxyamidine IDO1 inhibitors. The *in vivo* data show that INCB023843 and INCB024360 can inhibit kynurenine levels in plasma in multiple species and importantly, in tumors and TDLNs. To our knowledge, this is the first time that systemic reductions in tryptophan catabolism by a small molecule in tumors and in nonrodent species have been shown. Data reported for D-1MT, a described IDO2 inhibitor currently in clinical development, indicate that there was no de-

crease in plasma kynurenine levels in nonhuman primates (24) or in patients in early clinical trials (27). Multiple groups have described differences between D-1MT and L-1MT, showing that it is, in fact, L-1MT that can both decrease kynurenine generation and restore impaired proliferation of several T cell subsets and that IDO1 drives tryptophan catabolism in human dendritic

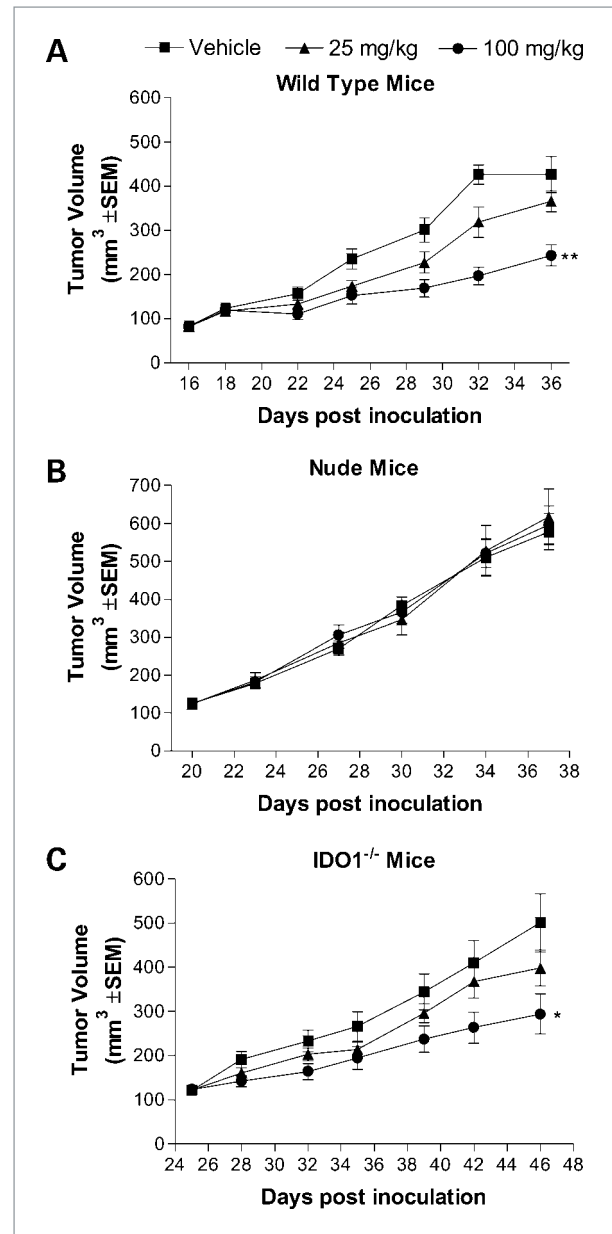


Figure 5. Lymphocytes are required for the *in vivo* activity of INCB023843. Female C57BL/6 mice bearing PAN02 tumors were treated orally twice daily with either vehicle (■) or INCB023843 at doses of 25 (▼) or 100 (●) mg/kg in (A) wild-type, (B) Balb/c *nu/nu*, or (C) *Ido1*^{-/-} mice. Mean tumor volumes (mm³) ± SE (*n* = 9 mice per group) are shown. Data are statistically significant for 100 mg/kg; **, *P* < 0.01, day 36, wild-type mice; *, *P* < 0.05, day 46, *Ido1*^{-/-} mice.

cells (28, 29). Hydroxyamidines, active against IDO1, suppress kynurenine generation in cells and *in vivo* and are therefore suitable to test the hypothesis that IDO1 inhibition may provide clinical benefit to cancer patients.

INCB023843 and INCB024360 were efficacious in multiple mouse models of cancer as single agents and, notably, in models where high IDO expression is prevalent and correlated with poor prognosis in human cancers. The single-agent activity of these compounds is uncommon among immunologic agents, such as anti-CTLA4, where combinations with cytotoxic agents, radiation therapy, or cytokine-secreting tumor cell vaccines are required for efficacy (30–32). Despite the need for combinations to be effective in preclinical models, anti-CTLA4 has resulted in positive clinical outcomes in a low but significant number of patients as a single agent. As such, preclinical models may underpredict antitumor responses to immune modulating agents in humans.

The antitumor activity of hydroxyamidine IDO1 inhibitors is evident in *Ido1*-deficient mice but not in immunodeficient mice, confirming that *Ido1* expression by tumors is sufficient to block antitumor immune responses regardless of host expression of *Ido1*. These data also show that inhibition of tumor-generated tryptophan ca-

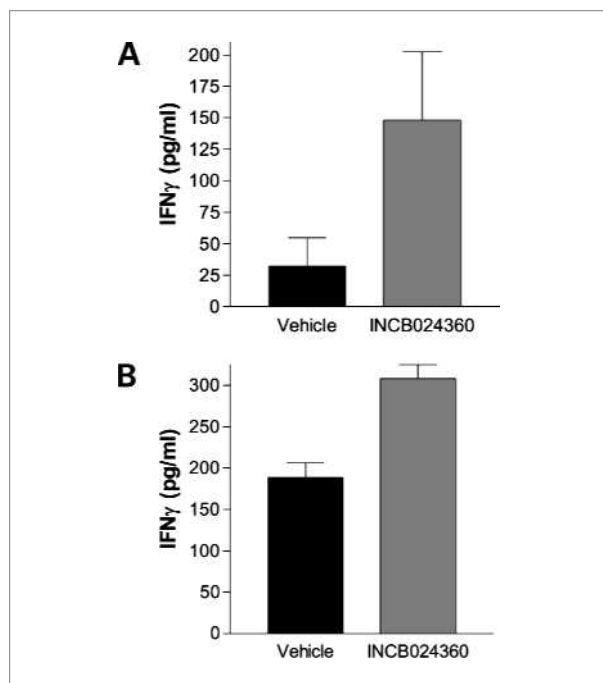


Figure 6. IDO inhibition enhances the number and activity of lymphocytes. A, TILs from CT26 tumors in Balb/c mice treated for 7 d with vehicle or INCB024360 (30 mg/kg orally twice daily) were cultured for 24 h and secreted IFN γ was measured by ELISA. Data are means \pm SD. B, lymphocytes from CT26 TDLNs in Balb/c mice treated for 7 d with either vehicle control or INCB024360 (30 mg/kg orally twice daily) were cultured for 24 h in the presence of 50 ng/mL phorbol 12-myristate 13-acetate plus 500 ng/mL ionomycin and secreted IFN γ was measured by ELISA. Data are mean \pm SD.

Table 2. INCB023843 increases the Teff/Treg ratio

	Vehicle	INCB023843
CD3 ⁺	19.4%	40.0%
CD4 ⁺	15.6%	26.1%
CD8 ⁺	8.1%	15.9%
CD4 ⁺ CD25 ⁺	16.0%	4.5%
CD8 ⁺ /CD4 ⁺ CD25 ⁺ ratio	0.5	3.5

tabolism can be therapeutic. This provides support to the impact of IDO on efferent responses (33) and confirms that tumor growth control is dependent upon a functional immune system. Similarly, comparisons of growth rates of PAN02 tumors in different strains of mice were affected by the presence of lymphocytes and the amount of host *Ido1* expression: tumor doubling is fastest in immunodeficient mice and slowest in *Ido1*^{-/-} mice.² These data also suggest that a lack of host *Ido1* activity can, to some extent, impede tumor growth. Finally, we have shown that TILs are more prevalent and more active after treatment with an IDO1 inhibitor.

The data presented here, taken together with the exceptional safety profile of the compound in preclinical toxicology studies, support the evaluation of INCB024360 clinically in cancer patients where a therapeutic margin in excess of 30-fold might be expected. Patient populations for these studies should be enriched for those where tumor cell expression of IDO has been seen and is correlated with poor prognosis, as anecdotal clinical evidence for prolonged survival in patients in whom IDO expression is restricted to the vasculature and immune cells has been reported (34, 35). Designing a suitable trial for testing the utility of IDO1 inhibitors will be guided by the experience from the trials of other immunotherapeutic agents such as the CTLA4 blockers ipilimumab and tremelimumab. Assessments 12 weeks after the start of therapy may not optimally predict long-term durable responses due to the slower induction of antitumor activity by these agents (2, 36). As such, clinical investigators are proposing new criteria for the evaluation of immunotherapeutic agents (37), allowing patients who may initially progress, either with regard to lesion size or to the appearance of new metastatic lesions, to remain on study if there is evidence of stable or diminishing total tumor burden. It is reasonable to believe that IDO inhibition would fall in this category because in our studies there is evidence of slower responses early after treatment commences, but stronger responses at later time points (see Fig. 3B, 30 mg/kg dose, days 10–17; Fig. 5A, both doses, days 16–18). Notably, this delayed initial response was

² H.K. Koblish and M.J. Hansbury, unpublished results.

also seen with anti-CTLA4 in mice bearing established B16-BL6 tumors in which tumor growth control was only seen approximately 10 days after administration of the granulocyte macrophage colony-stimulating factor secreting B16-BL6 cell vaccine (31). Fortunately, measurement of kynurenine levels in plasma samples from patients could prove to be a useful pharmacodynamic marker, similar to our preclinical assessments. It would provide us with a preliminary indication of the systemic activity of INCB024360 and can be viewed as a surrogate for affecting tryptophan catabolism in the tumor and its microenvironment.

In summary, our results suggest that potent and selective orally bioavailable IDO1 inhibitors can impede the growth of IDO-expressing tumors through the reduction of kynurenine in the microenvironment and enhancement of T cell function. Clinical trials in-

vestigating the activity of INCB024360 are therefore warranted.

Disclosure of Potential Conflicts of Interest

All authors are employees and stock shareholders of Incyte Corporation.

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