

Zinc Protoporphyrin IX Stimulates Tumor Immunity by Disrupting the Immunosuppressive Enzyme Indoleamine 2,3-Dioxygenase

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Abstract

The tryptophan catabolic enzyme indoleamine 2,3-dioxygenase (IDO) has emerged as an important driver of immune escape in a growing number of cancers and cancer-associated chronic infections. In this study, we define novel immunotherapeutic applications for the heme precursor compound zinc protoporphyrin IX (ZnPP) based on our discovery that it is a potent small-molecule inhibitor of IDO. Inhibitory activity was determined using *in vitro* and in-cell enzyme assays as well as a novel *in vivo* pharmacodynamic system. An irreversible mechanism of inhibition was documented, consistent with competition for heme binding in newly synthesized cellular protein. siRNA methodology and an IDO-deficient mouse strain were used to verify the specificity of ZnPP as an IDO inhibitor. In a preclinical model of melanoma, ZnPP displayed antitumor properties that relied on T-cell function and IDO integrity. ZnPP also phenocopied the known antitumor properties of IDO inhibitors in preclinical models of skin and breast carcinoma. Our results suggest clinical evaluation of ZnPP as an adjuvant immunochemotherapy in chronic infections and cancers in which there is emerging recognition of a pathophysiologic role for IDO dysregulation. *Mol Cancer Ther*; 9(6); 1864–71. ©2010 AACR.

Introduction

The immune system suppresses cancer by recognizing and eliminating nascent neoplastic cells as foreign entities. However, this process of immune surveillance also imposes a selection for the emergence of neoplastic cells that can escape immune destruction. Thus, through immune selection, cells within a subclinical tumor may evolve immune escape mechanisms that can promote the formation of a progressive malignancy. A central prediction of this immunoediting hypothesis is that tumor cells will adopt escape mechanisms that engender a tolerogenic microenvironment (1). Indeed, it is becoming increasingly clear that tumors co-opt tolerance mechanisms normally used to control responses to self-antigens or environmental antigens. Moreover, many of these tolerogenic mechanisms may thwart therapy. Thus, therapeutic outcomes may be improved by directly attacking immune escape

mechanisms as a tactic to derepress the powerful antitumor potential of the immune system (2). Accumulating evidence points to activation of the indoleamine 2,3-dioxygenase (IDO) pathway as a common immune escape mechanism in human cancers (3, 4). Two IDO enzymes have been described, the well-characterized IDO1 enzyme and the more recently reported IDO2 enzyme, both of which function in tryptophan catabolism. The role played by IDO1 in suppressing T-cell immunity is widely documented in numerous settings, including in cancer, in which there is broad evidence that its activation mediates tolerance to tumor antigens (5).

The catalytic activity of IDO enzymes relies on a heme prosthetic group. Heme levels are controlled by heme oxygenase-1 (HO-1), which catabolizes heme and thereby restricts its availability. Notably, HO-1 and IDO1 resemble each other in being able to exert T-cell immunosuppressive effects that are regulated by interferon- γ (IFN- γ) and linked to generation of induced T regulatory cell (Treg) activities promoting immune escape in cancer (6, 7). Further, the effects of HO-1 loss do not reflect an intrinsic loss of Treg function but rather a disabling of regulatory antigen-presenting cells that recruit Treg-suppressive capacity (6). These parallels prompt the hypothesis that HO-1 may constitute part of an IDO pathway of immune escape in cancer. In considering this hypothesis, we explored whether the HO-1 inhibitor zinc protoporphyrin IX (ZnPP), a heme precursor compound, might phenocopy the effects of experimental IDO inhibitors in their ability to stimulate immune-dependent antitumor responses. In support of the notion that it

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may offer oncological utility, ZnPP has been reported to exert cytotoxic effects that can inhibit tumor growth (8–11). In this report, we offer evidence that ZnPP exerts anti-tumor properties that can be mediated mainly by an immune-mediated mechanism that is causally based on IDO inhibition. Briefly, we found that ZnPP directly and irreversibly inhibits the catalytic activity of the distinct IDO1 and IDO2 enzymes in cell-based assays and that HO-1 targeting is nonessential for this inhibition. Thus, whereas ZnPP is a direct inhibitor of IDO enzymes and HO-1, it exerts immune-based therapeutic effects that are primarily linked to IDO inhibition. Our findings prompt clinical evaluation of ZnPP as a novel immunotherapy to degrade IDO-mediated barriers to immune surveillance that are established in a growing number of immune disorders, chronic infections, and cancers.

Materials and Methods

Antibodies

Goat anti-human HO-1 was purchased from Santa Cruz Biotechnology. Polyclonal rabbit antisera to IDO1 or IDO2 polypeptides were raised by a commercial supplier (Covance). IDO2 antiserum was raised with a mixture of recombinant murine and human IDO2 polypeptides produced in Rosetta *Escherichia coli* and purified using a GST-Trap column (GE Healthcare). Antibodies were screened for reactivity against the immunizing antigen by enzyme-linked immunosorbent assay and Western blotting. Samples with the highest titers were purified by affinity chromatography as follows: Serum was preabsorbed to a protein column containing glutathione S-transferase (GST); then, the GST-unbound fraction was passed over a GST-IDO1 column. The unbound material collected from this column was then affinity purified on a GST-IDO2 column containing both human and mouse recombinant his₆-tagged IDO2. The resulting affinity-purified polyclonal antibody was analyzed by Western blotting and confirmed to be IDO2 specific with no cross-reactivity to IDO1. In a similar fashion, IDO1 antiserum was raised by using a mixture of murine and human recombinant IDO1-V5-his₆ proteins produced in *E. coli*, yielding affinity-purified polyclonal antibody that was confirmed to be IDO1 specific with no cross-reactivity to IDO2. For immunoblotting, cellular lysate or protein samples were denatured in Laemmli sample buffer and resolved on SDS-polyacrylamide gels. After transferring to nitrocellulose, gel immunoblot analysis was done using a primary antibody at dilutions described in the figure legends. IDO1, IDO2, and HO-1 antibodies were detected using a 1:10,000 dilution of anti-rabbit (IDO1, IDO2) or anti-goat (HO-1) IgG conjugated to horseradish peroxidase (BD Biosciences). Detection was achieved using the ECL-Plus chemiluminescent system (Amersham Pharmacia Biotech) and visualized using KODAK Image Station 4000R (Carestream Health, Inc.).

Cell culture

All cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (Hyclone) and 1% penicillin-streptomycin (Invitrogen) at 37°C in 5% CO₂. Human 293-T-REx cell lines stably expressing IDO1 and IDO2 that have been described previously (12) were maintained in blasticidin (5 ng/mL) and zeomycin (25 µg/mL) and induced to express the transgene by the addition of doxycycline to 20 to 100 ng/mL. siRNA transfections were carried out as follows: Hu-HO1-specific siRNAs were purchased from Santa Cruz Biotechnology and transfected into HeLa or 293-T-REx cells stably expressing IDO1 and IDO2 (12) using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. The day after transfection, cells were stimulated as noted in the figure legends; 48 hours later, the culture medium was harvested for kynurenine assay and cells were harvested, lysed, and processed for Western blot analysis or Ni⁺ affinity pull-down assays. Cells seeded onto 96-well tissue culture plates at 25% confluency were treated 24 hours later with serial dilutions of ZnPP (Sigma), and sulforhodamine B cell viability assay was done after 72 hours of compound exposure. Briefly, cells were fixed for 1 hour with 10% trichloroacetic acid at 4°C, washed five times with water, air-dried, and stained with 0.4% (w/v) sulforhodamine B (Sigma) dissolved in 1% acetic acid. Unbound dye was removed by four washes with 1% acetic acid, plates were air-dried, and then the protein-bound dye was extracted with 10 mmol/L unbuffered Tris base (pH 10.5) for 5 minutes. Optical density was read at 510 nm using a Synergy HT microtiter plate reader. Using this assay, little cytotoxicity or growth suppression was observed in B16-F10 melanoma cells or HeLa cells with ZnPP exposures up to 100 µmol/L.

IDO enzyme assays

muIDO1 was purified from an inducible 293-T-REx cell line (12) after no treatment or treatment for 48 hours with 20 µmol/L ZnPP or the known pan-IDO inhibitor MTH-Trp (Sigma). Briefly, cells from two to three confluent 10-cm dishes were harvested for each condition, washed in PBS, and disrupted by sonication (5× 10-second burst at 40% intensity) in 50 mmol/L potassium phosphate buffer (pH 6.5). Cell lysates were clarified by centrifugation (12,000 rpm, 20 min), and supernatants were incubated with 50 µL of equilibrated Ni-NTA His Resin (EMD) for 60 minutes at 4°C. Beads were then collected and washed five times with 50 mmol/L potassium phosphate buffer. huIDO1 was purified from *E. coli* strain BL21DE3pLysS transformed with pet5Ahis₆huIDO1 by sequential chromatography over phosphocellulose and Ni-NTA agarose columns as described (13). *In vitro* enzyme activity was determined in reaction mixtures that contained 50 mmol/L potassium phosphate buffer (pH 6.5), 40 mmol/L ascorbic acid, 400 µg/mL catalase, and 20 mmol/L methylene blue. Activity was assessed for each IDO1 preparation, and the amount of enzyme used in the assay was based on this determination. The substrate, L-tryptophan (100 mmol/L

stock in 0.1 N HCl), was serially diluted from 200 to 25 $\mu\text{mol/L}$. Inhibitors were dissolved in DMSO to make 100 mmol/L stock solutions and assessed at final concentrations of 100 and 50 $\mu\text{mol/L}$ in a total reaction volume of 200 μL . Reactions were carried out at 37°C for 60 minutes, stopped by adding 30% (w/v) trichloroacetic acid to 3% final volume, and then heated at 65°C for 15 minutes to convert *N*-formyl-kynurenine to kynurenine. Plates were then spun at 6,000 $\times g$ for 5 minutes, and 100 μL supernatant from each well were transferred to a new 96-well plate and mixed with Ehrlich's reagent (2% *p*-dimethylaminobenzaldehyde w/v in acetic acid). The yellow color generated from the reaction with kynurenine after 10 to 30 minutes was quantitated at 490 nm using a Synergy HT microtiter plate reader (Bio-Tek). Data were analyzed by using Prism 4 software (Graph Pad). Cellular inhibitory activity of ZnPP was assessed against the human and mouse IDO1 enzymes stably expressed in 293-T-REx cells in a 96-well assay as described (12). Briefly, ZnPP solubilized in DMSO was serially diluted into cell cultures in plates that were then sealed in plastic wrap and incubated for 16 hours at 37°C in a humidified CO₂ incubator. To measure kynurenine, 200 μL of cell culture supernatants were mixed in a 96-well dish with 12.5 μL 30% TCA, incubated 30 minutes at 50°C, and clarified by 10 minutes of centrifugation at 3,000 to 10,000 rpm. Supernatants were processed as above on a plate reader (BioMek), and data were collected and analyzed using the Excel software (Microsoft). Samples were analyzed in triplicate with control values averaged and subtracted from experimental sample values.

Tumor treatment studies

Mice were bred at the Lankenau Institute in Opti-mouse vented cages and were free of common mouse pathogen infections. All experimental procedures done on mice were approved by the Lankenau Animal Care and Use Committee. C57BL/6 mice and athymic NCr-nu/nu (nude mice) mice were obtained from National Cancer Institute-Frederick. C57BL/6.*Ido1*^{-/-} mice (14) were a gift of A. Mellor, Immunotherapy Center, Medical College of Georgia, Augusta, GA. Treatment studies in B16-F10 melanomas and myc+ras transformed Bin1^{-/-} keratinocytes (MR KEC) were carried out in tumor isograft settings as described (15, 16). Briefly, 1 $\times 10^5$ cells were injected s.c. into male syngeneic C57BL/6J, C57BL/6J.*Ido1*^{-/-}, or nude mice. Treatment was initiated at day 7 after tumor cell engraftment by i.p. delivery of 20 mg/kg ZnPP in sterile saline twice daily for 5 consecutive days. Briefly, cells were injected s.c. into male syngeneic C57BL/6J, C57BL/6J.*Ido1*^{-/-}, or nude mice. Treatment was initiated at day 7 after tumor cell engraftment by i.p. delivery of 20 mg/kg ZnPP in sterile saline twice daily for 5 consecutive days. Treatment studies in spontaneous mammary tumor-bearing MMTV-neu mice were carried out in combination with paclitaxel as described (17). Tumor growth was monitored by caliper measurements of orthogonal diameters, and the estimated

tumor volume was calculated based on the formula for determining a prolapsed ellipsoid ($d^2 \times l/0.52$), where *d* is the shorter of the two orthogonal measurements. Graphing and statistical analysis of the data were done with GraphPad Prism 4 software. All *P* values shown were based on comparative evaluations using a two-tailed Student's *t* test.

Pharmacodynamic assay

IDO was induced in lungs of 8- to 10-week-old C57BL/6 mice by intrapulmonary delivery of lipopolysaccharides (LPS) from *E. coli* 0111:B4 (Sigma). Briefly, mice anesthetized by inhalation of isoflurane were instilled intranasally with 25 μg of LPS in 25 μL sterile saline. ZnPP was administered by i.p. injection at 20 mg/kg twice daily in 100 μL sterile saline, immediately after the LPS challenge, and with repeated dosing at 6 and 24 hours postchallenge. Mice were euthanized at 24 hours postchallenge by cervical dislocation, and lung tissues were weighed and frozen on dry ice. For analysis of kynurenine using the assay described above, frozen lung tissue samples were homogenized in PBS (1:4 w/v) and subjected to three freeze-thaw cycles of lysis. Deproteinized lysates were analyzed by high-performance liquid chromatography coupled to electrospray ionization tandem mass spectrometry in a Varian 320-MS triple quadrupole mass spectrometer system, as described in more detail elsewhere (18), with quantitation of kynurenine based on analysis of two daughter ions.

Results

IDO1 and immune competency are crucial for the antitumor properties of ZnPP

Several studies have reported that ZnPP or pegylated derivatives of this compound exhibit antitumor properties in preclinical mouse model systems (8–11). To investigate our hypothesis of an immune basis for ZnPP action, we used the B16-F10 murine melanoma graft model, which is well established for cancer immunotherapy studies. B16-F10 cells lack detectable IDO1 expression; thus, the host is the sole source of IDO1 activity in this model (19, 20).⁶ This design feature was important because it allowed us to compare ZnPP activity in a tumor-bearing animal in which the hypothesized target IDO1 was completely absent. Treatment was initiated 7 days after s.c. challenge with 1 $\times 10^5$ tumor cells. By this point, all engrafted mice had developed small palpable tumors of $\sim 100 \text{ mm}^3$ in a tight range of sizes (data not shown). The murine hosts used included isogenic C57BL/6J wild-type mice, *Ido1*^{-/-} C57BL/6J mice (*Ido*^{-/-} mice), or immunocompromised athymic nude (nu/nu) mice. In these experiments, ZnPP was administered i.p. once daily at 20 mg/kg for 5 consecutive days

⁶ R. Metz, J.B. DuHadaway, and G.C. Prendergast, unpublished observations.

only during the second week of the experiment. This dose lies at the upper end of the dose response range for antitumor activity documented in the literature (11). Consistent with previous reports (8–11), we observed that as a single agent, ZnPP suppressed B16 melanoma outgrowth in wild-type C57BL/6J mice. In stark contrast, ZnPP was largely inactive in *Ido*^{-/-} hosts lacking the presumptive target (Fig. 1A). In support of these observations, we found that ZnPP displayed little or no *in vitro* cytotoxicity against B16-F10 melanoma cells (IDO nonexpressing) or HeLa cells (IDO expressing) when tested at concentrations of up to 100 $\mu\text{mol/L}$, respectively (Supplementary Fig. S1).

Additional experiments established the specificity and extent of the antitumor effects of ZnPP. To assess specificity relative to other protoporphyrins, we compared the activity of the clinical agent SnPP, which is similar to ZnPP except that it contains tin instead of zinc in the protoporphyrin backbone. Under conditions of the same route and schedule of administration, SnPP lacked antitumor activity (Supplementary Fig. S2). We then compared the effects of ZnPP in skin carcinoma tumors produced by isografts of *Bin1*^{-/-} MR KECs, a population of oncogenically transformed keratinocytes that express IDO1 and form tumors in an IDO-dependent manner (17). Under conditions of the same dose, route,

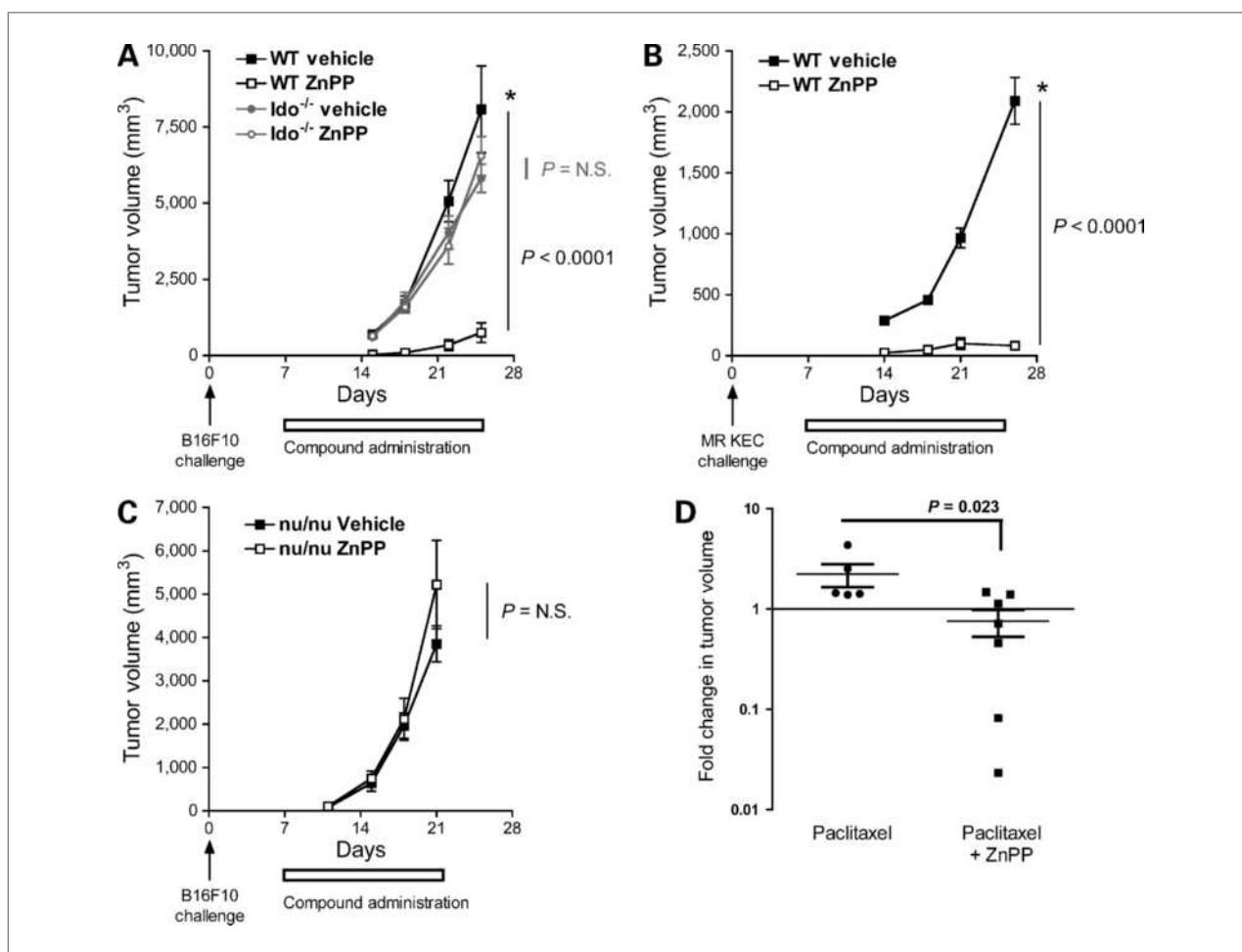


Figure 1. IDO1 targeting and immune competence are crucial for ZnPP antitumor effects. **A**, antitumor effects of ZnPP against B16 melanoma are nearly abolished by IDO deletion. C57BL/6J wild-type (WT) or *Ido*^{-/-} mice were engrafted with 1×10^5 B16-F10 cells and 7 days later were treated with control vehicle only or with ZnPP (20 mg/kg) injected i.p. once daily for 5 consecutive days. Caliper measurements of tumors were recorded biweekly with mean and SEM plotted for each group [$n = 7$, except wild-type ZnPP ($n = 10$) and *Ido*^{-/-} ZnPP ($n = 6$)]. N.S., not significant. **B**, ZnPP inhibits the growth of MR KEC skin carcinoma. Wild-type mice were engrafted with 1×10^7 MR KECs and 7 days later were treated with vehicle or ZnPP and analyzed as above ($n = 5$). **C**, adaptive immunity is critical to ZnPP antitumor effects. Athymic NCr nude mice (nu/nu) were engrafted with B16-F10 cells and treated with vehicle or ZnPP as above. Caliper measurements of tumors were recorded biweekly with mean and SEM plotted for each group (vehicle, $n = 4$; ZnPP, $n = 5$). **D**, ZnPP cooperates with paclitaxel to inhibit the growth of MMTV-neu breast carcinoma. Mice bearing tumors of $\sim 0.5 \text{ mm}^3$ were enrolled in a 2-week trial as described (17) comparing paclitaxel response in the absence or presence of ZnPP (20 mg/kg). Each point on the graph represents a single tumor measurement with mean and SEM shown for each group.

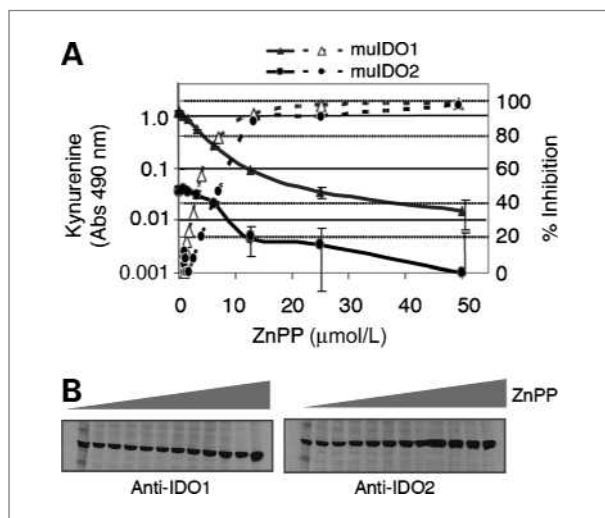


Figure 2. ZnPP inhibits the activity of IDO1 and IDO2 in cells. **A**, enzyme assays. 293-T-REx cells expressing mulDO1 or mulDO2 were incubated in the presence of varying concentrations of ZnPP (0–50 μmol/L). Kynurenine levels were measured after 48 hours (solid lines) and the percent inhibition was plotted on the same graph (dashed lines). IC_{50} values were calculated from the linear portion of the curve with mulDO1 IC_{50} = 3.3 μmol/L and mulDO2 IC_{50} = 7.1 μmol/L. Abs, absorbance. **B**, Western blot analysis. Levels of IDO1 and IDO2 protein in the extracts analyzed above were evaluated using anti-IDO1 and anti-IDO2 antibodies. The results shown correspond to wells treated with varying levels of ZnPP to assess IDO1 and IDO2 protein levels during the cellular treatment course. The image represents the exposed radiographic film scanned over a Ponceau-S-stained blot to confirm similar protein loading and blot transfer.

and schedule of administration, ZnPP potently inhibited tumor formation by MR KECs (Fig. 1B). In contrast, ZnPP was unable to inhibit B16 tumor outgrowth in immunocompromised nude mice in which T-cell function is compromised, with treated tumors in these mice even slightly larger at the experimental end point (Fig. 1C). The B16 and MR KEC isograft tumor assays each used a design in which ZnPP treatment was initiated against palpable but relatively small tumors of ~100 mm³. This was appropriate to test the hypothesis that ZnPP is an IDO inhibitor because IDO inhibitors are not efficacious in treating large tumors by themselves, unless combined with chemotherapy (15, 17, 21). To assess the efficacy of ZnPP against larger tumors, we used an aggressive model of autochthonous breast carcinoma, MMTV-neu transgenic mice, in which combination treatment with an IDO inhibitor cooperates with paclitaxel to yield tumor regression (15, 17, 21, 20). In this model, ZnPP administration at the same dose, route, and schedule phenocopied the characteristic ability of an IDO inhibitor to elicit regression of larger established tumors (~500 mm³) at the start of the treatment (Fig. 1D). Thus, ZnPP acted like other IDO inhibitors in its ability to leverage the effect of chemotherapy. We concluded that ZnPP exerted antitumor effects through a mechanism that relied on competent immunity and IDO1 targeting.

ZnPP inhibits the enzymatic activity of IDO1 and IDO2 in cells

IDO enzymes require heme as a prosthetic group for their dioxygenase activity. ZnPP is known as a competitive inhibitor of HO-1, which degrades heme. However, the notion that the antitumor effects of ZnPP related directly to HO-1 inhibition was counterintuitive because inhibition of HO-1 by ZnPP would increase rather than decrease heme availability for use in the *de novo* synthesis of IDO enzymes. We therefore reasoned that ZnPP would directly inhibit IDO1 and/or the related enzyme IDO2, the activity of which may rely on IDO1 integrity (4, 12). This line of reasoning was supported by the finding that IDO1 was required for ZnPP to manifest its antitumor effects, due to the absence of a critical target, as is the case for certain other IDO inhibitors (15, 20, 21). In further support, ZnPP inhibited the catabolic activity of the murine IDO1 and IDO2 enzymes expressed in 293-T-REx cells with similar IC_{50} values of 3 to 7 μmol/L (Fig. 2). Thus, ZnPP efficiently inhibited both IDO enzymes. We confirmed the findings in HeLa cells treated with IFN-γ, in which the activity of the endogenously induced human IDO1 enzyme was suppressed by ZnPP at the same

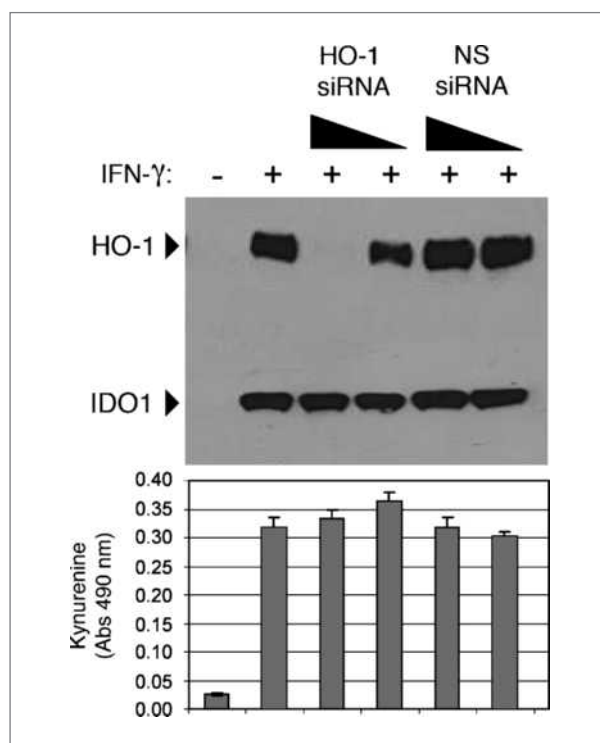


Figure 3. HO-1 is not essential to the ability of ZnPP to inhibit IDO activity in cells. Endogenous HO-1 and IDO expression in parallel with IDO activity that is induced by IFN-γ in HeLa cells was assessed by Western blot analysis or kynurenine assay. Untreated or IFN-γ-treated cells were untransfected or transfected with human HO-1-specific or nonspecific siRNAs (40 or 10 pmol/L) before cells were harvested for analysis. Cell lysates were prepared in radioimmunoprecipitation assay buffer 48 hours after IFN-γ treatment and siRNA transfection.

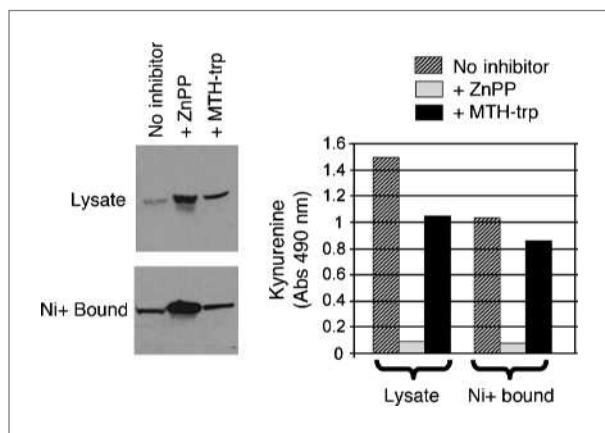


Figure 4. ZnPP irreversibly inhibits the activity of newly synthesized IDO enzyme. His₆-epitope-tagged huIDO1 was purified from induced 293-T-REx cells that were untreated or treated with 20 μ mol/L ZnPP or 20 μ mol/L of the substrate competitive pan-IDO inhibitor MTH-trp (12, 17). Inhibitors were added to cell cultures but not to subsequent *in vitro* analysis of enzyme purified from cells (such that only an irreversible inhibition would persist). Western blot analysis documented similar levels of huIDO1 in all three treatment groups, with most of the cellular enzyme retained on the Ni⁺ affinity column (left). Equivalent amounts of eluted material from Ni⁺ affinity columns were used to assay enzyme activity by *in vitro* reaction (right). Blots were stained with Ponceau-S to confirm equivalent protein loading and gel transfer.

potency (data not shown). ZnPP acted specifically insofar as the related tin-containing compound SnPP completely lacked activity as an IDO inhibitor (data not shown). We concluded that ZnPP acted selectively to disrupt IDO activity by acting at the level of newly synthesized enzyme in cells.

To rule out the possibility that ZnPP indirectly affected IDO activity by directly blocking HO-1 activity, we investigated whether knocking down HO-1 expression inhibited IDO activity. HO-1 and IDO1 are both inducible in HeLa cells that are stimulated by IFN- γ . Therefore, we used a siRNA-based strategy to specifically repress HO-1 expression under these conditions. The specific siRNAs we used suppressed IFN- γ -induced expression of HO-1 >90% relative to a nonspecific siRNA (Fig. 3). In siRNA-transfected cells, IDO1 expression and activity was unaffected whether or not the expression of HO-1 was knocked down. Furthermore, we saw little to no effect of supplementing heme or adding the heme precursor aminolevulinic acid to IFN- γ -treated HeLa cells under the experimental conditions (data not shown). Taken together, these results supported the interpretation that ZnPP did not act indirectly through HO-1 inhibition, but instead proximally by inhibiting IDO activity directly.

To define the level at which ZnPP acted on IDO, we compared its effects on purified enzyme activity versus its effects on *de novo* synthesized enzyme in cells. Against recombinant IDO1 enzymes that were purified from bacteria or insect cells, ZnPP lacked inhibitory activity at a large range of concentrations tested (Sup-

plementary Fig. S3). This observation ruled out the possibility that ZnPP substituted with the heme prosthetic group after the enzyme was translated and folded into its native conformation. It remained possible that ZnPP could stably substitute for the heme group during protein folding, thereby leading to the production of a mature but inactive enzyme. To determine whether ZnPP acted at this level to interfere with production of the *de novo* synthesized enzyme (i.e., by competing with the incorporation of the heme group as the enzyme folded), we compared the activity of a his₆-epitope-tagged IDO1 enzyme purified by nickel affinity chromatography from untreated or ZnPP-treated 293-T-REx cells in which the enzyme was induced (Fig. 4). Consistent with this expectation, we found that under these conditions of purification, IDO was efficiently inhibited by ZnPP. An apparent elevation in steady-state IDO protein levels in this experiment was not found to be reproducible (data not shown). In contrast to the effects of ZnPP, IDO1 was not irreversibly inhibited by a competitive inhibitor (MTH-trp), the effects of which are reversible after enzyme purification from cells. Furthermore, we observed that the effects of ZnPP were not relieved by phosphatase treatment (data not shown), ruling out the possibility of an indirect allosteric effect mediated by enzyme phosphorylation *in vivo* that could be relieved (24). In summary, we concluded that ZnPP could directly and irreversibly inhibit IDO1 in cells at the level of the *de novo* synthesized enzyme.

ZnPP inhibits IDO activity *in vivo*

Using a lung-based mouse pharmacodynamic assay, we confirmed that ZnPP could effectively inhibit IDO under conditions that were associated with the antitumor properties of this agent. Pulmonary exposure to bacterial LPS endotoxin significantly increased IDO protein level and enzymatic activity in lung tissue isolated from wild-type mice, but not from *Ido1*^{-/-} mice, demonstrating the specificity of this assay for IDO1 function (18). In tissues isolated from animals euthanized 24 hours after intranasal instillation of LPS, we observed an ~2-fold increase in kynurenine levels relative to the levels in unexposed mice (Fig. 5). Under these conditions, ZnPP administration was sufficient to suppress the level of kynurenine in lung 77% relative to the LPS-induced level, or 26% above the level of the uninduced control (Fig. 5). We concluded that the IDO1-dependent antitumor properties of ZnPP were associated with its ability to achieve a significant inhibition of IDO activity *in vivo*.

Discussion

The findings of this study suggest novel applications for ZnPP in clinical oncology based on its IDO-inhibitory properties. Antitumor effects reported previously for

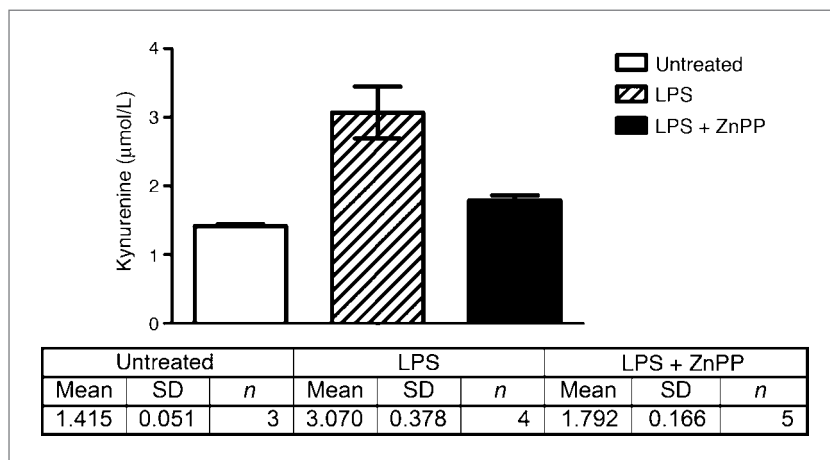


Figure 5. ZnPP inhibits IDO *in vivo*. Lung tissue was harvested for analysis from C57BL/6J mice that were euthanized 24 hours after intranasal instillation with 25 μ g LPS. In the experimental group, 25 μ g ZnPP was administered i.p. at 0, 6, and 24 hours after LPS instillation. Kynurenine levels in tissue homogenates were determined by electrospray ionization tandem mass spectroscopy. The SEM is shown for the data in each treatment group.

ZnPP have been interpreted to reflect its ability to inhibit HO-1, the catabolites of which (bilirubin and biliverdin) are powerful scavengers of reactive oxygen species that might explain the cytoprotective action of HO-1. Evidence offered in this report suggests that ZnPP can exert antitumor effects through disruption of IDO-mediated immune escape in cancer. ZnPP might be applied against earlier-stage tumors or to leverage the effect of chemotherapy against larger tumors; further experiments to examine dose and route or schedule of administration may optimize these possible applications. Like the IDO enzymes, HO-1 may support the activation of regulatory T-cell functions that mediate immune escape. However, whether HO-1 inhibition participates in immune responses in cancer has not been addressed.

We found that the antitumor properties of ZnPP were largely abolished in the absence of IDO1 and that ZnPP could inhibit IDO activity in a HO-1-independent manner. These findings speak genetically and biochemically to the concept that the antitumor properties of ZnPP may be based to a significant degree on a disruption of IDO signaling. Our findings do not rule out the possibility that HO-1 inhibition by ZnPP could play some role in facilitating an immune response or in decreasing the levels of cytoprotective HO-1 reaction products bilirubin and biliverdin. Nevertheless, whereas HO-1 inhibition may be involved in certain therapeutic settings, in the absence of IDO1, our results indicate that HO-1 inhibition may not be sufficient. In contrast to another report (11), we did not observe any correlation between antitumor and cytotoxic activities, the latter of which was minimal for ZnPP in both the B16-F10 melanoma cells and HeLa cells we used in this study. Although the basis is unknown, melanoma cells have not been examined by others, and differences in cellular susceptibility to ZnPP cytotoxicity are readily conceivable. Nevertheless, at a minimum, our findings establish that the antitumor properties of ZnPP extend beyond cytotoxicity to involve immune-based and IDO-targeted

effects that are efficacious on their own. Thus, ZnPP may exert at least two antitumor activities, one related to cytotoxicity and a second to relieving IDO-mediated immune escape.

Targeting tumoral immune suppression represents a novel approach to cancer treatment; however, this area has yet to be vigorously pursued in part due to a paucity of suitable agents to advance. Relative to other IDO-inhibitory compounds that have been described, ZnPP has unique and appealing properties for clinical evaluation. It has a unique mechanism of action that targets both IDO enzymes as well as HO-1. It is closely related to the clinical agent SnPP, suggesting mimicry for the clinical development pathway and cues for safety monitoring. Last, as a heme precursor, it occurs naturally *in vivo* as the first clinically translatable inhibitor of both IDO1 and IDO2. ZnPP has been used widely as a tool in pediatric and newborn testing for lead paint poisoning and hyperbilirubinemia, respectively (24). Evaluating ZnPP based on its IDO inhibitory properties offers an opportunity to reposition this compound as an immunochemotherapeutic agent that limits immune escape in cancer. Our findings offer initial support for the notion that ZnPP might be applicable where IDO is expressed in either the tumor or the host immune system. Preclinical evidence supports the combination of IDO inhibitors with "immunogenic" chemotherapeutic drugs (15, 17). Although one study has reported negative findings on the combinatorial use of ZnPP with chemotherapy (11), this issue should be reconsidered further, given the opportunity ZnPP may provide to improve outcomes in specific settings of combinatorial immunochemotherapy where IDO inhibitors are effective (17). In considering these properties, we also propose ZnPP as an immunochemotherapeutic agent to treat diseases beyond cancer, which are characterized by local or systemic immune suppression, where IDO dysregulation occurs and has been implicated in persistent pathology (e.g., chronic infections).

Disclosure of Potential Conflicts of Interest

R. Metz, J.B. DuHadaway, D.H. Munn, A.J. Muller, M. Mautino, and G.C. Prendergast each disclose intellectual property interests, receive income, and/or have financial holdings in NewLink Genetics Corporation, a biotechnology company developing IDO inhibitors for cancer treatment.

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