Identification and Characterization of a Novel Dual Inhibitor of Indoleamine 2,3-dioxygenase 1 and Tryptophan 2,3-dioxygenase

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ABSTRACT: Kynurenine (Kyn), a metabolite of tryptophan (Trp), is a key regulator of mammalian immune responses such as cancer immune tolerance. Indoleamine-2,3-dioxygenase (IDO) and tryptophan 2,3-dioxygenase (TDO) are main enzymes regulating the first and rate-limiting step of the Kyn pathway. To identify new small molecule inhibitors of TDO, we selected A172 glioblastoma cell line constitutively expressed TDO. Characterization of this cell line using kinase inhibitor library resulted in identification of MEK/ERK pathway-dependent TDO expression. After knowing the properties for TDO expression, we further proceeded to screen chemical library for TDO inhibitors. We previously determined that S-benzylisothiourea derivatives are enzymatic inhibitors of indoleamine 2,3-dioxygenase 1 (IDO1) and suggested that the isothiourea moiety could be an important pharmacophore for binding to heme. Based on this premise, we screened an in-house library composed of various isothiourea derivatives and identified a bisisothiourea derivative, PVZB3001, as an inhibitor of TDO. Interestingly, PVZB3001 also inhibited the enzymatic activity of IDO1 in both cell-based and cell-free assays but did not inhibit other heme enzymes. Molecular docking studies suggested the importance of isothiourea moieties at the ortho position of the phenyl ring for the inhibition of catalytic activity. PVZB3001 showed competitive inhibition against TDO, and this was supported by the docking simulation. PVZB3001 recovered natural killer (NK) cell viability and functions by inhibiting Kyn accumulation in conditioned medium of both IDO1- and TDO-expressing cells. Furthermore, oral administration of IDO1-overexpressing tumor-bearing mice with PVZB3001 significantly inhibited tumor growth. Thus, we identified a novel selective dual inhibitor of IDO1 and TDO using the Kyn production assay with a glioblastoma cell line. This inhibitor could be a useful pharmacological tool for modulating the Kyn pathway in a variety of experimental systems.

KEYWORDS: Tryptophan, kynurenine, indoleamine-pyrrole 2,3-dioxygenase (IDO1), tryptophan 2,3-dioxygenase (TDO), mitogen-activated protein kinase kinase (MEK), dual inhibitor, immunotherapy, drug screening

Introduction

Tryptophan (Trp) is catabolized mainly by the kynurenine (Kyn) pathway. Indoleamine 2,3-dioxygenase 1 and 2 (IDO1 and IDO2) and tryptophan 2,3-dioxygenase (TDO) catalyze the oxidative cleavage of the indole ring of L-Trp and convert it to N-formyl Kyn in the Kyn pathway.1 IDO1 is expressed widely in many tissues such as lung, placenta, lymphoid tissue, and inflammatory lesions.2 TDO is expressed in liver, kidney tubules, and the reproductive tract.3 TDO is predominantly expressed in liver and brain and maintains the homeostasis of L-Trp in the liver.4,5 Tumors have multiple mechanisms to suppress the immune system and promote cancer progression. Upregulation of Trp catabolism accompanied with the expression of IDO1 and TDO in the tumor microenvironment is an important mechanism for immune tolerance and to enhance cancer progression.6,7 Thus, IDO1 and TDO are attracting much attention as drug targets.

Kyn is a key regulator of immune tolerance and suppresses allogeneic T-cell proliferation by acting as an endogenous ligand of ary1 hydrocarbon receptor (AhR).8 AhR is a ligand-dependent transcriptional receptor and forms a heterodimer with AhR nuclear translocator that binds to the response element in the promoter regions of target genes such as interleukin-6 (IL-6) and IL-10. AhR regulates immune system-related cells, such as dendritic cells (DCs), macrophages, natural killer (NK) cells, innate lymphoid cells, type 17 helper T-cells, type 22 helper T-cells, and regulatory T-cells (Tregs).5 IDO1 is expressed in various human tumors such as cervical carcinoma, endometrial carcinoma, kidney carcinoma, and non-small cell lung carcinoma.2 Many studies have revealed the mechanisms of IDO1 expression by using tumor or immune cells. Janus kinase 1 (JAK1), JAK2, and signal transducers and activators of transcription 1 (STAT1) activated by interferon-γ (IFN-γ) lead to transcription of IDO1 via STAT1 binding to
the IFN-γ-activated site (GAS) in the IDO1 promoter.\textsuperscript{9,10} Additionally, AhR-IL-6-STAT3 signaling maintains constitutive expression of IDO1.\textsuperscript{11} Kyn activates AhR and promotes the production of IL-6. IL-6 secreted from the tumor binds to its receptor and induces STAT3 phosphorylation and IDO1 transcription. Taken together, there is an apparent positive feedback loop involving IDO1, Kyn, and AhR. Cancer cells use this signaling loop to suppress the immune response and acquire resistance toward anticancer drugs, including immune checkpoint inhibitors.\textsuperscript{5}

In contrast, TDO is expressed in glioblastoma, colorectal carcinoma, bladder carcinoma, lung carcinoma, hepatocellular carcinoma, and breast carcinoma.\textsuperscript{8,12,13} In renal cell carcinoma, TDO expression is associated with Tregs infiltration and correlates with resistance to immune checkpoint inhibitor treatment.\textsuperscript{14} Moreover, the TDO knock-out in mice was reported to increase the efficacies of anti-CTLA4 antibody and anti-PD1 antibody by keeping Trp concentration high in blood.\textsuperscript{15} The regulation mechanism of TDO in cancer cells is poorly understood, although TDO expression in normal cells is reported to be activated by Trp, glucocorticoid, heme, NAD(P)H negative feedback, and prostaglandin E2 (PGE2).\textsuperscript{5,16,17}

Various types of IDO1 inhibitors have been so far reported\textsuperscript{18} and several IDO1 inhibitors are in clinical development, such as epacadostat, navoximod, and BMS-986205. On the other hand, several TDO inhibitors and IDO1/TDO dual inhibitors have been also reported.\textsuperscript{15,19-22} However, there have not been yet clinically therapeutic drugs approved for cancer treatment.

The present study aimed to identify new small molecule inhibitors of TDO. Using a kinase inhibitor library, we found that the glioblastoma cell line A172 constitutively expresses TDO in a mitogen-activated protein kinase (MEK)/extracellular-signal-regulated kinase (ERK) signaling pathway-dependent manner. We screened an in-house chemical library using A172 and identified a bisisothiourea derivative, PVZB3001, as a selective and cell-permeable dual inhibitor of IDO1 and TDO. PVZB3001 showed stronger inhibition of Kyn production than did a selective IDO1 and TDO inhibitor in both IDO1- and TDO-expressing cells, respectively. Furthermore, PVZB3001 recovered NK cell viability and function by inhibiting Kyn accumulation in the conditioned medium of both IDO1- and TDO-expressing cells. PVZB3001 could be a useful pharmacological tool for modulating the Kyn pathway in a variety of experimental systems. The discovery and biochemical characterization of PVZB3001 are presented in this report.

Materials and Methods

Reagents and antibodies

rhTDO and rhIDO1 proteins were purchased from BPS Bioscience (San Diego, CA) and L-Trp and IFN-γ were obtained from Sigma-Aldrich (St. Louis, MO). The kinase library, 680C91, trametinib, TAK-733, AZD8330, and epacadostat were purchased from Selleck Biotech (Tokyo, Japan), Tocris Bioscience (Bristol, UK), ChemScene (Monmouth Junction, NJ), AdooQ Bioscience (Irvine, CA), ChemieTek (Indianapolis, IN), and MedChemExpress (Monmouth Junction, NJ), respectively. PVZB3001, PVZB3056 and PVZB3057 were purchased from Sigma-Aldrich. The compounds were dissolved in DMSO. Anti-TDO antibody was purchased from Merck Millipore (Cat. No. MABN1537; Burlington, MA) and anti-IDO1 antibody was from Oriental Yeast (Cat. No. 47150000; Tokyo, Japan). Anti-ERK1/2 antibody (Cat. No. 4695) and anti-p-ERK1/2 (Thr202/Thr204) antibody (Cat. No. 4370) were purchased from Cell Signaling Technology (Danvers, MA). Anti-α-tubulin antibody (Cat. No. T9026) was purchased from Sigma-Aldrich. Anti-mouse IgG, horseradish peroxidase linked F(ab’)2 fragment (Cat. No. NA9310) and Anti-rabbit IgG, horseradish peroxidase linked F(ab’)2 fragment (Cat. No. NA9340) were purchased as second antibodies from Cytiva (Tokyo, Japan).

Cell lines and culture

Cell lines A431 (human squamous cell carcinoma) and NK-92MI (human malignant non-Hodgkin’s lymphoma) were purchased from ATCC (Manassas, VA). A172 (human brain glioblastoma) and HeLa (human adenocarcinoma) were purchased from JCRB (Osaka, Japan) and Riken BioResource Research Center (Tsukuba, Japan), respectively. A431 cells, A172 cells and HeLa cells were maintained in Dulbecco’s Modified Eagle Medium with high glucose supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 50 U/mL penicillin, and 50 μg/mL streptomycin. NK-92MI cells were maintained in Alpha Minimum Essential medium supplemented with 0.2 mM inositol, 0.1 mM 2-mercaptoethanol, 0.02 mM folic acid, horse serum at a final concentration of 12.5%, heat-inactivated FBS at a final concentration of 12.5%, and 50 U/mL penicillin, and 50 μg/mL streptomycin. Cells were maintained at 37°C in 5% CO₂.

Cell-based Kyn production assay

Cells were seeded in 96-well plates and incubated for 24 hours. After treatment with the test compounds for 1 hour, L-Trp was added and the cells were cultured for 24 hours. The final concentration of L-Trp in the A431 and A172 culture medium was 178 μM and 524 μM, respectively. For IDO1 expression in A431 cells and A172 cells, IFN-γ was added at the same time as Trp. The final concentration of IFN-γ in each medium was 10 ng/mL. To each well, 200 μL of mixed solution of 7% (v/v) aqueous CCl₃COOH and 2% (w/v) p-dimethylaminobenzaldehyde in acetic acid (2.5) was added. The absorbance derived from Kyn was measured at 460 nm with a SpectraMax microplate reader (Molecular Devices, Sunnyvale, CA).
Cell viability assay

Cells were seeded in 96-well plates and incubated for 24 hours. After treatment with the test compounds for 1 hour, L-Trp was added and the cells were cultured for 24 hours. The final concentration of L-Trp in the A431 and A172 culture medium was 178 μM and 524 μM, respectively. For IDO1 expression in A431 cells and A172 cells, IFN-γ was added at the same time as Trp. The final concentration of IFN-γ in the medium was 10 ng/mL. Water-soluble tetrazolium salt (WST-8) reagent (Cell Counting Kit-8, Dojindo, Kumamoto, Japan) was added to each well and the plates were incubated for 40 to 60 minutes at 37°C. The bio-reduction of WST-8 reagent was monitored by measuring absorbance at 450 nm with a SpectraMax microplate reader.

Enzyme assay

rhTDO protein (0.8 μg/mL) and rhIDO1 protein (0.3 μg/mL) were separately incubated with test compounds (2% DMSO) in the assay buffer (50 mM potassium phosphate buffer (pH 6.5), 20 mM L (+)-ascorbic acid (pH 7.0), 10 μM methylene blue, and 100 μg/mL catalase from bovine liver) for 10 minutes at 37°C. L-Trp (200 μM for IDO1 and 2 mM for TDO) was added and the reaction solution (total volume 200 μL) was incubated for 120 minutes at 37°C. After 40 μL of 30% (v/v) aqueous CCl₃COOH was added, the solution was heated for 15 minutes at 50°C. Next, 150 μL of supernatant was transferred to new 96-well plates and 150 μL of 2% (w/v) p-dimethylaminobenzaldehyde was added to the wells. The absorbance was measured at 490 nm with a SpectraMax microplate reader. For calculation of the IC₅₀ value for each inhibitor, data from 3 independent duplicate experiments were pooled, and dose-response curves were fitted using GraphPad PRISM5 (MDF, Tokyo, Japan).

Western blot analysis

Cultured cells were lysed in RIPA buffer (10X RIPA buffer and protease inhibitor cocktail, Cell Signaling Technology). Cell lysates (20-30 μg of protein/lane) were subjected to electrophoresis on a polyacrylamide gel and then transferred to a Trans-Blot Turbo PVDF membrane (Bio-Rad Laboratories, Hercules, CA). Membranes were blocked with 5% skim milk or Blocking Reagent for Can Get Signal (TOYOBO, Osaka, Japan) and incubated with primary antibody overnight at 4°C. The membranes were incubated with secondary antibody for 1 hour at room temperature. Immunoblots were visualized using ECL Prime Western Blotting Detection Reagent (Cytiva) with an LAS-3000 (Fujifilm, Tokyo, Japan) and ImageQuant LAS 500 imager (Cytiva).

Real-time reverse transcription PCR (qRT-PCR)

Total RNA was purified using NucleoSpin RNA (Takara Bio, Shiga, Japan) according to the manufacturer’s instructions, and 500 ng of total RNA from each sample was used to synthesize the first cDNA strand. cDNA was prepared using a PrimeScript RT reagent kit (Takara Bio). Real-time reverse transcription-PCR (qRT-PCR) was carried out following the manufacturer’s protocol for Premix Ex Taq (Takara Bio) with a PikoReal Real-Time PCR system (Thermo Fisher Scientific, Waltham, MA). Three technical replicates were run for each gene in each sample. TaqMan Gene Expression Assay Probes for TDO (Hs00194611_m1), ERK1 (Hs00385075_m1), ERK2 (Hs01046830_m1), MEK1 (Hs00605615_mH), MEK2 (Hs00360961_m1), and β-actin (Hs01060665_g1) were purchased from Thermo Fisher Scientific. Relative quantification was carried out using the 2⁻ΔΔCT method. The mRNA expression level was normalized against the housekeeping gene β-actin.

RNA interference

Cells were transfected with each siRNA (ON-TARGET plus; Horizon Discovery, Cambridge, UK) using Lipofectamine RNAiMAX (Thermo Fisher Scientific). Cells were diluted with growth medium without antibiotics. siRNA and transfection reagent were diluted in serum-free Opti-MEM and mixed. Following incubation at room temperature for 5 minutes, the mixture was added to the wells of 96-well plate and then the cell suspension was seeded at 15 000 cells/well. The final siRNA concentration was 20 nM. After 48 hours following siRNA transfection, medium was changed to fresh medium, then after 24 hours cell pellets were collected for qRT-PCR. The catalog numbers of the ON-TARGET plus SMARTpool (Thermo Fisher Scientific) targeting each gene are as follows: human ERK1 (L-003592-00-0005), human ERK2 (L-003555-00-0005), human MEK1 (L-003571-00-0005), human MEK2 (L-003573-00-0005), human TDO (L-008506-01-0005), and non-targeting (D-001810-10-20). Non-targeting siRNAs were used as controls.

Docking studies

Docking studies were carried out using MOE 2019.0102 software (MOLSIS, Tokyo, Japan). The three-dimensional structures of IDO1 and TDO were obtained from the protein data bank (PDB id: 5xe1 for IDO1, 6pyy and 5tia for TDO). In docking analysis using MOE, the structure was hydrogenated using the Protonate 3D module. After partial charges were assigned using an all-atom force field combining AMBER1024 and Extended Hückel Theory, hydrogen atoms were minimized, followed by removal of the original ligands. The Alpha Site Finder module26 was used to define the ligand binding site. PVZB3001 and PVZB1890 generated by the stochastic search method were docked on the binding site. Docked poses were optimized by the AMBER10: EHT force field combining AMBER1024 and Extended Hückel Theory,25 hydrogen atoms were minimized, followed by removal of the original ligands. The Alpha Site Finder module26 was used to define the ligand binding site. PVZB3001 and PVZB1890 generated by the stochastic search method were docked on the binding site. Docked poses were optimized by the AMBER10: EHT force field, then ranked according to the GBVI/WSA scoring function,27 which estimates the free energy of binding of the ligand from a given pose. The interaction was analyzed using the top ranking in each docking.

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Kinetics analysis

Kinetics analysis of IDO1 and TDO activity was carried out following the enzyme assay method mentioned above. The concentrations of the substrate, L-Trp, were 20, 40, 60, and 120 µM for IDO1 and 300, 500, 750, 1500, and 3000 µM for TDO. Each compound was used at the following concentrations: 0.5 and 1 µM for 680C91, 15 and 30 µM for 1-MT, 1 and 2 µM for PVZB3001. The initial reaction velocities were calculated from 4 time points (4, 8, 12, and 16 minutes) at each substrate concentration. The mode of inhibition was determined using Lineweaver–Burk plot analysis, in which the inverse of the initial rate was plotted against the inverse of the substrate concentration in the presence or absence of test compounds.

Proliferation assay and cell killing activity of NK cells

A172 cells were seeded in 96-well plates and incubated for 24 hours. Medium was changed to 500 µM L-Trp-containing culture medium of NK-92MI and test compounds were added to the wells. After treating for 1 hour with the test compound, IFN-γ was added to each well. The final concentration of IFN-γ was 10 ng/mL. Cells were cultured for 48 hours and then the supernatant of the culture medium was collected as conditioned medium. NK-92MI cells were seeded in 96-well plates and cultured in the conditioned medium. After 72 hours, NK-92MI cells were used for cell proliferation, cell killing activity. In the cell proliferation assay, CellTiter-Glo (Promega, Madison, WI) was used to evaluate the cell viability. In the supernatants, the granzyme B concentration was measured using a human Granzyme B ELISA kit (Diaclone, Besancon, France), according to a protocol recommended by the manufacturer.

Cell line establishment for in vivo study

The murine cancer cell lines CT26 (colon carcinoma) were obtained from ATCC. Ido1 overexpressing CT26 cell line (CT26-Ido1) was established by transduction of CT26 with the Ido1 gene in pMXs-Puro Retroviral Vector (Cell Biolabs, San Diego, CA, Cat. No. RTV-012). Clonal transformants were selected using 5 µg/mL puromycin. Cells were maintained in RPMI medium supplemented with 2 mM L-Glutamine, 10% heat-inactivated FBS and 50 U/mL penicillin, and 50 µg/mL streptomycin and 1 mM L-Tryptophan. Expression of Ido1 was confirmed by quantifying Ido1 mRNA in the total mRNA extracted from cells by qRT-PCR. The catalog numbers of the TaqMan Gene Expression Assay Probes (Thermo Fisher Scientific) targeting each gene are as follows: Ido1 (Mm00492586_m1), Gapdh (Mm99999915_g1).

Evaluation of antitumor activity in vivo

The antitumor efficacy of PVZB3001 was investigated in a mouse syngeneic model. Female BALB/c mice aged 6 weeks were purchased from Japan SLC, Inc. (Shizuoka, Japan), 1 x 10^6 CT26-Ido1 cells were inoculated subcutaneously into the right and left dorsal region of the mice. 6 days after transplantation, they were randomly allocated to the following groups (n = 8): a vehicle group; a PVZB3001 monotherapy group (12.5 mg/kg); a PVZB3001 monotherapy group (25 mg/kg); and a PVZB3001 monotherapy group (50 mg/kg). Treatment was started on day 6; the test compounds were administered orally once a day. Tumor growth was monitored until day 14 by measuring 2 perpendicular diameters every 2 days using a digital caliper (Mitutoyo, Kanagawa, Japan), and tumor volume was calculated as follows. V = (W² x L)/2 (V: volume; W: Minor axis; L: Major axis). On day 14, tumors were weighed. The antitumor efficacy was expressed based on tumor weight at day 14 as the percentage tumor growth inhibition (% IR), which was calculated using the formula: IR (%) = (1 - mean weight of the treated tumor/mean tumor weight in the vehicle group) x 100. Body weight was monitored every 2 days to assess the tolerability of the therapy. The relative body weight at day n was calculated using the following formula: Relative body weight = body weight on day n/body weight on day 1. Tumors were harvested and assayed to measure mRNA expression level and Kyn/Trp ratio. For mRNA expression level, tumors were dissociated by gentleMACS Dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany) in Lysis buffer of NucleoSpin RNA and RNA extraction, cDNA synthesis, and qRT-PCR were performed as described above. For Kyn/Trp ratio, tumors were dissociated by gentleMACS Dissociator (Miltenyi Biotec) in PBS and the concentrations of Kyn and Trp were measured by kynurenine ELISA kit (ImmunoSmol, Bordeaux, France, BA E-2200) and Tryptophan ELISA kit (ImmunoSmol, BA E-2700), respectively, according to the manufacturer’s instructions.

Ethics statement

All animal experiments were conducted at the University of Shizuoka Animal Center with the approval of the Steering Committee in accordance with the University of Shizuoka Animal Experiment Guidelines.

Statistical analysis

Statistical analysis data were expressed as mean ± SD. Statistical analysis was performed using the Welch’s t test for 2 independent groups or Dunnett’s Test for multiple comparison
to a single control. A P-value of <.05 was considered as statistically significant.

Results

Constitutive expression of TDO is dependent on the MEK/ERK pathway in A172 cells

We previously developed a cell-based high-throughput screening (HTS) assay using Ehrlich's reagent to identify new types of small molecules that regulate Kyn production. We conducted this assay on 21 cell lines to identify a cell line that constitutively produces Kyn without cytokine stimulation (Figure 1A, Supplemental Table 1) and determined that the glioblastoma cell line A172 produces Kyn at relatively high levels. This cell line expresses TDO but not IDO1, and Kyn production was reduced by the selective TDO inhibitor 680C91 (data not shown). We confirmed that Kyn production in A172 cells was still in linear phase at 24 hours (Supplemental Figure 1). It is important to understand the regulatory mechanism for the constitutive expression of TDO in A172 cells prior to enzyme inhibitor screening using this cell line. For this purpose, we conducted a cell-based Kyn production assay with A172 cells against a kinase inhibitor library containing 270 distinct small molecules that target a large variety of human protein kinases. We identified 10 compounds that exhibited ≦20% Kyn production and ≧80% cell viability as primary hits (Figure 1B). To eliminate non-specific inhibitors and cytotoxic compounds, we subjected the 10 hit compounds to a cell-based Kyn production assay and Cell viability assay in A431 cells expressing IDO1 upon IFN-γ stimulation. We chose 5 compounds that resulted in ≧80% Kyn production and ≧80% cell viability in A431 cells and all of them were MEK inhibitors (Supplemental Table 2). The selective effect of MEK inhibitors on Kyn production in A172 cells suggests that the MEK signaling pathway might regulate TDO-dependent Kyn production. We focused on the 3 hit MEK inhibitors trametinib, TAK-733 and AZD8330 that most potently inhibited Kyn production in A172 cells. In association with the inhibition of ERK1/2 phosphorylation (Figure 1C), trametinib decreased Kyn production in a dose-dependent manner without reducing cell viability, with an inhibitory activity at 200 nM similar to that of 5 μM 680C91 (Figure 1D). Trametinib did not inhibit recombinant human TDO (rhTDO) enzymatic activity in the cell-free assay, in contrast to 680C91 (Figure 1E). We then assessed the effect of trametinib on TDO expression in A172 cells. Trametinib downregulated both mRNA and protein expressions at 100 and 1000 nM, respectively (Figure 1F and G). The MEK inhibitors TAK-733 and AZD8330 attenuated Kyn production with their reduction of TDO expression (Supplemental Figure 2), suggesting that the reduction of TDO-dependent Kyn production by MEK inhibitors may be associated with the downregulation of TDO expression in A172 cells. Next, we evaluated the contribution of the MEK/ERK pathway to the TDO expression levels under knockdown of MEK1/2, ERK1/2, and TDO by siRNA treatment. Each siRNA significantly decreased its target mRNA expression level in A172 cells (Supplemental Figure 3). Under these conditions, all siRNAs significantly decreased the TDO mRNA expression level, although with various degrees of knockdown efficiency (Figure 1H). The effect of MEK1/ERK1 siRNA was more prominent than that of MEK2/ERK2 siRNA. Taken together, we demonstrated for the first time that constitutive high expression of TDO is dependent on the activated MEK/ERK pathway in A172 cells. The results also suggest that kinase inhibitors of the MEK/ERK pathway might be obtained as false positive hits during the screening of direct enzyme inhibitors using this assay.

Identification of the bisisothiourea derivative PVZB3001 as a dual inhibitor of IDO1 and TDO

We previously identified the S-benzylisothiourea derivative PVZB1890 (compound 3d in reference 31) as an IDO1 enzyme inhibitor by screening using A431 cells treated with IFN-γ. We anticipated that the isothiourea moiety of PVZB1890 might be an important pharmacophore for interaction with the heme in the catalytic domain of IDO1. TDO also contains heme, and thus we expected to identify direct TDO inhibitors by screening a variety of isothiourea derivatives. We screened an in-house chemical library composed of isothiourea derivatives using a cell-based Kyn production assay with A172 cells and identified a bisisothiourea derivative, PVZB3001 (Figure 2A). PVZB3001 strongly inhibited Kyn production in A431 cells stimulated with IFN-γ (Figure 2A). PVZB3001 inhibited cellular Kyn production dose-dependently in A172 cells and in A431 cells upon IFN-γ stimulation (Figure 2B), with an IC_{50} value of 0.96 μM in A172 cells and 0.74 μM in A431 cells. These results suggest that PVZB3001 inhibits IDO1 in addition to TDO in cells. Thus, we selected this compound for further evaluation and investigated its pharmacological mode of action.

PVZB3001 exhibited selective and direct inhibition of IDO1 and TDO

The chemical structures of PVZB1890 and PVZB3001 are shown in Figure 3A. PVZB3001 has 2 isothiourea moieties at the ortho position. We used a cell-free assay to assess the direct inhibitory activity of PVZB3001 against rhTDO and rhIDO and observed dose-dependent inhibition of both proteins, with an IC_{50} value of 0.62 μM for rhTDO and 1.71 μM for rhIDO (Figure 3A and Table 1). We confirmed that PVZB3001 did not change the protein expression levels of TDO and IDO1 (Figure 3B). There was no or only a slight effect on other heme enzymes such as inducible nitric oxide synthase (iNOS) and cytochrome p450 (CYP) (Supplemental Table 3). The bisisothiourea derivatives PVZB3056 and PVZB3057 have isothiourea moieties at the meta and para positions, respectively (Figure 3A) and show no inhibitory activity against rhTDO and rhIDO1 (Figure 3A and Table 1).
Figure 1. Assessment of the contribution of the MEK/ERK pathway to TDO-dependent Kyn production in A172 cells. (A) The relative ability of Kyn production in 21 cell lines. The levels of Kyn production were determined by Ehrlich reaction 24 hours after addition of L-Trp (final concentration 524 μM) to the cultured cells. (B) The scatter plot shows cell viability (x-axis) and Kyn production (y-axis) from the results of the kinase library screening (270 compounds, final concentration 10 μM). The hit compounds are shown as red data points. The levels of Kyn production were determined by Ehrlich reaction 24 hours after addition of L-Trp (final concentration 524 μM) to the cultured cells. The compounds were added 1 hour before addition of L-Trp. (C) Western blot analysis of A172 cells treated with the indicated concentration of trametinib. The analysis was performed 24 hours after addition of L-Trp (final concentration 524 μM) to the cultured cells. Trametinib was added 1 hour before addition of L-Trp. α-tubulin was used as a loading control. (D) Kyn production and cell viability in A172 cells treated with the indicated concentration of trametinib. 680C91 was used as a positive control. The levels of Kyn production were determined by Ehrlich reaction and cell viability was determined by WST-8 reagent 24 hours after addition of L-Trp (final concentration 524 μM) to the cultured cells. Trametinib was added at the indicated concentrations 1 hour before addition of L-Trp. (E) Effect of trametinib on enzymatic activity of rhTDO. 680C91 was used as a positive control. The levels of Kyn production were determined by Ehrlich reaction. (F) Relative TDO mRNA expression level in A172 cells treated with the indicated concentration of trametinib. The analysis was performed 24 hours after addition of trametinib to the cultured cells. α-tubulin was used as a loading control. (G) Relative TDO mRNA expression level in A172 cells transfected with siRNAs targeting MEK/ERK signaling. The mRNA expression level was quantified by qRT-PCR 72 hours after siRNA transfection. Non-targeting siRNA was used as a control and siTDO was used as a positive control. Data are representative of 3 independent experiments and values are expressed as the means ± SD of triplicate samples. **Indicates P < .01 using Dunnett's test comparing to DMSO (D-F) or control (H).
We next performed in silico molecular docking to investigate the possible binding mode of PVZB3001 with TDO and IDO1, respectively. PVZB1890 was also subjected to the molecular docking. The published crystal structure of TDO (PDB id: 6pyy) was chosen as the template for docking because of its relatively high resolution. As for IDO1, the crystal structure of IDO1 in complex with INCB14943 (PDB id: 5xe1) was chosen because of its ligand similarity with the isothiourea substructure amidine and phenyl ring Cl atom of PVZB1890.

As shown in Figure 3C, the phenyl ring of PVZB3001 was located in the hydrophobic space in the heme pocket of both proteins. The NH of the isothiourea group formed a hydrogen bond with the propionic acid of the heme in both proteins. In the interaction with TDO, the NH of the 2 separate isothiourea groups formed a hydrogen bond with Ser155 and Glu80 (Figure 3C). Similar interactions were observed in IDO1, with the NH of the isothiourea groups forming hydrogen bonds with Ser263 or Ser167 (Figure 3C). The docking pose of PVZB1890 with TDO showed hydrogen bonds between the NH of the isothiourea group and the heme propionic acid and Ser263, and a halogen bond between the Cl atom on the phenyl ring and Cys129 in the hydrophobic space (Figure 3D). As for PVZB3001, it also showed hydrogen bonds between the NH of the isothiourea group and the heme propionic acid and Ser263, and a halogen bond between the Cl atom on the phenyl ring and Cys129 in the hydrophobic space (Figure 3D). These results support the potential utility of PVZB3001 as an IDO1 and TDO dual inhibitor and that of PVZB1890 as an IDO1 selective inhibitor.

**PVZB3001 showed competitive inhibition against TDO**

Kinetics studies were performed to determine the inhibition patterns of PVZB3001. First, we confirmed that 680C91 and L-1-methyltryptophan (L-1MT) showed competitive inhibition against TDO and IDO1 by Lineweaver–Burk plot analysis, respectively, as reported previously (Figure 4A and B). The inhibition patterns against IDO1 were different for the 2 concentrations tested (Figure 4C, Supplemental Figure 4A). In contrast, the inhibition patterns against IDO1 were different for the 2 concentrations tested (Figure 4D, Supplemental Figure 4B). Like PVZB1890, PVZB3001 also exhibited non-competitive inhibition at 1.0 μM but exhibited...
Figure 3. Biochemical characterization of the bisisothiourea derivative PVZB3001 as a dual inhibitor of IDO1 and TDO. (A) Chemical structures and the dose-dependent inhibitory activities against rhTDO and rhIDO1 of PVZB1890, PVZB3001, PVZB3056 and PVZB3057. Graphs were drawn using Xlfit (ITOCHU Techno-Solutions Corporation, Tokyo, Japan). Data are representative of 3 independent experiments and values are expressed as the means ± SD of triplicate samples. (B) Western blot analysis of A172 cells expressing TDO and A431 cells expressing IDO1 treated with the indicated concentration of PVZB3001. The analyses were performed 24 hours after addition of L-Trp (final concentration 524 μM in A172 cells and 178 μM in A431 cells) to the cultured cells. PVZB3001 was added 1 hour before addition of L-Trp. IFN-γ (final concentration 10 ng/mL) was added to A431 cells for IDO1 expression at the same time as Trp. α-tubulin was used as a loading control. Data are representative of 3 independent experiments. (C and D) The docking poses of (C) PVZB3001 (colored pink in ball-stick representation) and (D) PVZB1890 (colored violet in ball-stick representation) in TDO and IDO1. The key residues, including HEME, are shown as green sticks and hydrogen or halogen bonds are shown as dotted lines. The interaction mode was obtained through molecular docking (PDB id: 5xe1 for IDO1 and 6pyy for TDO) and depicted using MOE 2019.0102.
mixed inhibition at 2.0 μM. Mixed inhibition comprises a mixture of competitive and noncompetitive inhibition and occurs when an inhibitor can bind either to the free enzyme or to the enzyme-substrate complex but with different affinities. To better understand the mechanism behind the competitive inhibition of PVZB3001 against TDO, we performed a docking analysis in the same manner as described above using the co-crystal structure of Trp-TDO (PDB id: 5tia). As shown in Figure 4E and F, the phenyl ring of PVZB3001 oriented with a pose similar to that of the indole ring of the native ligand Trp. In addition, the NH at the isothiourea group of PVZB3001, as well as the NH of Trp, formed hydrogen bonds with the propionic acid of the heme and Thr342. These docking results imply that PVZB3001 binds at the substrate binding site of TDO through an interaction similar to that of Trp, supporting the competitive inhibition results obtained for PVZB3001 against TDO.

**Table 1. IC<sub>50</sub> values of isothiourea derivatives of PVZB1890, epacadostat, and 680C91 on rhTDO and rhIDO1.**

| ENZYME   | IC<sub>50</sub> VALUE (μM) |
|----------|---------------------------|
| PVZB1890 |                           |
| PVZB3001 |                           |
| PVZB3056 |                           |
| PVZB3057 |                           |
| EPACADOSTAT |                     |
| 680C91   |                           |

PVZB3001 recovered NK cell viability and function by inhibiting Kyn accumulation in conditioned medium of both IDO1- and TDO-expressing cells

Both IDO1 and TDO are overexpressed in several tumor types. The inhibition of IDO1 and TDO provides an additive inhibitory effect on Kyn production in cells expressing both IDO1 and TDO, and thus we evaluated whether PVZB3001 inhibits Kyn production in cells expressing both of these proteins. A172 cells express TDO constitutively and also express IDO1 upon IFN-γ stimulation. Kyn production by A172 cells increased upon IFN-γ stimulation, whereas TDO expression was unchanged (Figure 5A and B), suggesting that IDO1 expression increases Kyn production additively. Under our experimental conditions, PVZB3001 inhibited Kyn production significantly without reducing cell viability (Figure 5A) and PVZB3001 did not change the protein expression level (Figure 5B). Though the selective IDO1 inhibitor epacadostat and the selective TDO inhibitor 680C91 also decreased Kyn production, the inhibitory activity of PVZB3001 was higher than that of epacadostat and 680C91 at 3 μM or higher concentrations in cells expressing both IDO1 and TDO (Figure 5A).

Trp metabolites, including Kyn, inhibit T-cell and NK cell proliferation and function and thus we investigated whether PVZB3001 could recover the proliferation and function of NK cells by reducing Kyn production. First, we confirmed that Kyn reduced the viability of NK-92MI cells and granzyme B production dose-dependently, as previously reported (Supplemental Figure 5). Next, we collected conditioned medium from IDO1/TDO-expressing A172 cells treated with PVZB3001. The viability of NK-92MI cells cultured in the conditioned medium increased with PVZB3001 dose-dependently (Figure 5C). Additionally, we assessed whether the function of NK-92MI cells could be reversed when cultured in the conditioned medium. NK-92MI cells induced the death of HeLa cells in a PVZB3001 dose-dependent manner (Figure 5D) showing that PVZB3001 reduces Kyn production in A172 cells and recovers not only cell proliferation but also the cell killing activity of NK-92MI cells.

**PVZB3001 suppressed tumor growth in vivo**

Next, we investigate whether PVZB3001 exhibits antitumor effect in vivo. We established Ido1-overexpressing murine colon carcinoma cell line, CT26-Ido1 (Supplemental Figure 6A). The Tdo mRNA expression in CT26-Ido1 cells was not detected by qRT-PCR (data not shown). PVZB3001 inhibited Kyn production without reducing cell viability in CT26-Ido1 (Supplemental Figure 6B). Both tumor growth and Kyn/Trp ratio were elevated in the IDO1-overexpressing tumors (Supplemental Figure 6C and D). We treated mice bearing CT26-Ido1 cells orally with PVZB3001. Oral administration of PVZB3001 suppressed the growth of tumor in a dose-dependent manner without body weight loss, especially showed significant antitumor effect when treated with 25.0 mg/kg and with 50.0 mg/kg respectively (Figure 6A). The Kyn/Trp ratio was decreased in tumors from PVZB3001-treated mice (Figure 6B). On the other hand, oral administration of 50.0 mg/kg epacadostat under the same conditions showed moderate effect without statistical significance (Supplemental Figure 6E).

**Discussion**

The catabolism of Trp, mediated by IDO1 and TDO, is a key immunosuppressive pathway in many types of tumors. IDO1 is widely expressed in cancer cells, and thus many selective IDO1 inhibitors have been developed, some of which are under clinical development. Similar to IDO1, TDO suppresses antitumor immune responses and promotes tumor cell survival. Hence, identifying new chemotypes of TDO inhibitors is important for developing new cancer immunotherapies. Although several TDO inhibitors and IDO1/TDO dual...
Figure 4. Inhibition pattern of PVZB3001 against rhTDO and rhIDO1. (A and B) Lineweaver-Burk plot analysis of (A) rhTDO in the presence of 680C91 or (B) rhIDO1 in the presence of L-1MT and (C) rhTDO in the presence of PVZB3001 or (D) rhIDO1 in the presence of PVZB3001. DMSO was used as a control. The horizontal axis shows the reciprocal of the substrate concentration (1/s) and the vertical axis shows the reciprocal of the initial reaction velocity (1/v). Data are representative of 3 independent experiments. The other 2 independent Lineweaver-Burk plot analyses of each enzyme in the presence of PVZB3001 were shown in Supplemental Figure 4. (E and F) The docking poses of (E) PVZB3001 alone in TDO and (F) PVZB3001 and Trp in TDO. PVZB3001 is shown colored pink in ball-stick representation and Trp is shown colored cyan in ball-stick representation. The key residues, including HHEME, are shown as green sticks and hydrogen are shown as dotted lines. The interaction mode was obtained through molecular docking (PDB id: 5tia) and depicted using MOE 2019.0102.
Figure 5. Effect of Kyn inhibition by PVZB3001 on cell viability and cell killing activities of NK-92MI. (A) The dose-dependent inhibitory activities of PVZB3001, epacadostat and 680C91 against Kyn production and cell viability in A172 cells expressing IDO1 and TDO by IFN-γ stimulation. A172 cells were treated with the indicated concentrations of test compounds. The levels of Kyn production were determined by Ehrlich reaction and cell viability was determined by WST-8 reagent 24 hours after addition of L-Trp to the cultured cells. PVZB3001 was added 1 hour before addition of L-Trp (final concentration 524 μM) and IFN-γ (final concentration 10 ng/mL). Kyn production and cell viability in A172 cells treated with DMSO and IFN-γ were used as controls. (B) Western blot analysis of A172 cells expressing IDO1 and TDO by IFN-γ stimulation. A172 cells were treated with the indicated concentration of PVZB3001. The analysis was performed 24 hours after addition of L-Trp to the cultured cells. PVZB3001 was added 1 hour before addition of L-Trp (final concentration 524 μM) and IFN-γ (final concentration 10 ng/mL). α-tubulin was used as a loading control. (C) The change in cell viability of NK-92MI cells cultured in conditioned medium collected from A172 cells expressing IDO1 and TDO treated with the indicated concentration of PVZB3001 for 72 hours. Cell viability was determined using CellTiter-Glo. Medium shows the result when NK-92MI cells were cultured in their culture medium. Cell viability of NK-92MI cultured in conditioned medium collected from A172 cells treated with DMSO was used as a control. (D) The change in cell viability of HeLa cells co-cultured with NK-92MI cells cultured in conditioned medium collected from A172 cells treated with the indicated concentration of PVZB3001 for 5 hours. Cell viability was determined by WST-8 reagent. Medium shows the result when HeLa cells were co-cultured with NK-92MI cells in their culture medium. Cell viability of HeLa cells co-cultured with NK-92MI cells cultured in conditioned medium collected from A172 cells treated with DMSO was used as a control. Data are representative of 3 independent experiments and values are expressed as the means ± SD of triplicate samples. * and ** indicate P < .05 and P < .01, respectively, using Dunnett’s test comparing to control. ## indicates P < .01 using a Welch’s t test, in comparing to control.
inhibitors have been reported, there are few reports analyzing IDO1/TDO dual inhibitors in detail and there have not been yet clinically therapeutic drugs approved for cancer treatment. In this study, we characterized our novel IDO1/TDO dual inhibitor both in vitro and in vivo.

A kinase inhibitor library screening using A172 cells showed that MEK inhibitors attenuated TDO-dependent Kyn production by inhibiting TDO expression in A172 cells (Figure 1A-G). Indeed, knockdown of MEK1, MEK2, ERK1, and ERK2 reduced TDO expression (Figure 1H). Because trametinib, TAK-733 and AZD8330 inhibit both MEK1 and MEK2, the inhibition level of the TDO expression by each siRNA was lower than MEK inhibitors. Additionally, the TDO expression by knockdown differed between MEK1 and MEK2, and between ERK1 and ERK2 (Figure 1H). ERK1 and MEK1 are very similar to ERK2 and MEK2, respectively, and are functionally redundant, but several reports show different contributions of MEK1 and MEK2 in cell cycle progression, cell proliferation, and cell morphology and invasive ability in cancer cells. Similarly, previous studies have shown different functions of ERK1 and ERK2 in cell cycle progression and migration and invasion. Taken together, the contributions of MEK1 and MEK2, and ERK1 and ERK2 in regulating TDO expression in A172 cells may be different.

Moreover, because MEK inhibitors decreased Kyn production both in A172 and other cell lines such as MDA-MB-453 cells (data not shown), the TDO expression-regulating mechanism of the MEK/ERK pathway is likely common in some types of cancer cells. MEK/ERK signaling is upregulated by activating mutations of receptor tyrosine kinase (RTK), Ras, and Raf in cancer cells. MDA-MB-453 has Ras mutations and mitogen-activated protein kinase (MAPK) signaling is

Figure 6. PVZB3001 reduced Tumor volume in vivo model. (A) Tumor volume (left) and Body weight (right) in mice bearing CT26-Ido1 tumors treated with PVZB3001 (12.5, 25, or 50 mg/kg, n=8) or vehicle (n=8). (B) Kyn/Trp Ratios in tumors treated with PVZB3001 (12.5, 25, or 50 mg/kg, n=8) or vehicle (n=8) were calculated from the Kyn and Trp concentration in tumors measured by ELISA. Data are expressed as the means ±SD of triplicate samples. * and ** indicates P<.05 and P<.01, respectively, using Dunnett’s test comparing to vehicle.
activated. However, activating mutations in RTK, Ras, and Raf are unknown in A172 cells. A172 has a homozygous deletion in phosphatase and tensin homolog deleted on chromosome 10 (PTEN) and there are several reports that PTEN inhibits the activation of MEK/ERK pathway. Thus, the MEK/ERK pathway is likely activated by PTEN deletion and overexpresses TDO in A172 cells.

In the 21 cell lines we tested, the MEK/ERK pathway is activated in the A431, HepG2, and MDA-MB-468 cell lines but Kyn production is low (Figure 1A). These results suggest that the MEK/ERK signaling pathway could be necessary but not sufficient for the regulation of TDO expression. The present study did not reveal the regulation mechanism between the MEK/ERK signaling pathway and TDO expression. However, regulation between TDO and the PGE2/prostaglandin E receptor-4 (EP4) pathway, and between the MEK/ERK pathway and cyclooxygenase 2 (COX2) have been reported suggesting that the MEK/ERK pathway may regulate TDO expression through the COX2/PGE2/EP4 pathway. A previous report showed that the CCAAT/enhancer-binding protein β (C/EBPβ) binding site in the promoter of TDO is essential for constitutive TDO expression in glioblastoma cells. Because A431 and MDA-MB-468 cells either weakly express or do not express COX2 and HepG2 expresses COX2, C/EBP, and TDO, the expression of these factors may affect whether the MEK/ERK pathway regulates TDO expression or not. Further experiments are needed to elucidate the relationship of these factors with MEK/ERK signaling in regulating TDO expression.

TDO is a crucial factor mediating immune suppression in tumors and is an attractive therapeutic target for improving anti-tumor immunity. To our knowledge, this is the first report to reveal that the MEK/ERK signaling pathway regulates TDO expression in cancer cells. Our findings suggest that therapeutic intervention of MEK/ERK signaling may restore antitumor immune responses via TDO.

Next, we screened derivatives of an in-house IDO1 inhibitor and identified PVZB3001 as an IDO1/TDO dual enzyme inhibitor (Figures 2 and 3). Both IDO1 and TDO contribute to the malignancy of cancers and both enzymes are overexpressed in some tumors. Additionally, one reason for the clinical failures of selective IDO1 inhibitors in treating melanoma and renal cell carcinoma may be the complementation of TDO in the absence of IDO activity or endogenous expression of TDO. Therefore, dual inhibition of IDO1 and TDO could provide an enhanced therapeutic effect in cancers.

Molecular docking studies implied the interactions between the phenyl ring and 2 isothiourea groups in PVZB3001 and the substrate binding pockets of IDO1 and TDO (Figure 3C and D). These interactions may explain why PVZB3001 is a dual inhibitor of IDO1 and TDO, and why PVZB1890 is a selective IDO1 inhibitor. Bisothiourea derivatives including the 1,2-disubstituted derivative PVZB3001, were reported in 1994 as NOS inhibitors, but there have been no reports of IDO1 and TDO inhibition. It was previously reported that the regiosomer of PVZB3001, 1, 3-disubstituted derivative PVZB3056 and 1, 4-disubstituted derivative PVZB3057, exhibit NOS inhibitory activity. Therefore, enzyme assays against heme-containing proteins, including iNOS and CYP enzymes other than IDO1 and TDO, were performed to confirm the selectivity of PVZB3001 (Supplemental Table 3). PVZB3001 was found to be a highly selective inhibitor of IDO1 and TDO. Interestingly, PVZB3056 and PVZB3057 showed no inhibitory activity against IDO1 or TDO (Figure 3A, Table 1). These results indicated that proper positioning of the 2 isothiourea groups on the phenyl ring, as is the case with PVZB3001, is important for high selectivity for IDO1 and TDO inhibition.

Some isothiourea derivatives were reported to have inhibitory effect on Na/K-ATPase and DMT1. Inhibition of Na/K-ATPase induces cytotoxicity, and inhibition of DMT1 causes iron deficiency anemia. PVZB3001 against Na/K-ATPase was reported to be 14.2 μM and IC50 value against DMT1 was reported to be equal or greater than 1 μM. From these reports, inhibitory activities of PVZB3001 against Na/K-ATPase and DMT1 seems to be much weaker than IDO1 and TDO.

For IDO1 inhibitors, various inhibition patterns were reported, such as competitive inhibition with L-Trp, non-competitive inhibition by binding heme-iron of IDO1, and irreversible inhibition by targeting heme-free apo form of IDO1. On the other hand, there are few reports to analyze the inhibition patterns of IDO1/TDO dual inhibitors. Determination of the inhibition patterns is important for understanding the differences among the IDO1/TDO inhibitors. We, therefore, performed kinetics studies of PVZB3001. We showed that PVZB3001 inhibited TDO competitively using Lineweaver-Burk plot analysis (Figure 4A, Supplemental Figure 4A). Molecular docking studies supported this TDO-inhibition pattern against IDO1/TDO dual inhibitors. Although more detailed investigations including structural analysis of the drug-enzyme complex are needed to accurately determine the IDO1 inhibition pattern of...
PVZB3001, the current data provide new insights into structure-based drug design targeting IDO1/TDO.

Partial inhibition of Kyn production by 680C91 indicated that selective inhibition of TDO is insufficient for complete inhibition of Kyn production in cells expressing both IDO1 and TDO (Figure 5A). On the other hand, the selective IDO1 inhibitor epacadostat inhibited Kyn production more effectively in cells expressing both IDO1 and TDO (Figure 5A). Epacadostat inhibited the enzymatic activity of TDO at 1 μM or higher concentrations even though epacadostat has 100-fold selectivity toward IDO1 over TDO.\textsuperscript{77} (Table 1). We considered that epacadostat showed further inhibition of Kyn production at 1 μM or higher concentrations because of its dual inhibition of IDO1 and TDO. Since PVZB3001 showed stronger inhibition than epacadostat at the concentrations at which both inhibitors inhibit IDO1 and TDO, PVZB3001 could lead to improved treatment in tumors expressing both IDO1 and TDO. Kyn induces immunosuppression by suppressing the proliferation and function of CD4\textsuperscript{+} T-cells, CD8\textsuperscript{+} T-cells and NK cells by activating AhR.\textsuperscript{8} In this study, we focused on the effect of PVZB3001 on NK cells in vitro. The proliferation and killing activity of NK-92MI was significantly recovered by the conditioned medium from PVZB3001-treated A172 cells in which both IDO1 and TDO were expressed (Figure 5C and D). We further explored the in vivo anti-tumor activity of PVZB3001 using mice bearing CT26-Ido1 cells. Oral administration of PVZB3001 suppressed both tumor growth and Kyn/Trp ratio in a dose-dependent manner (Figure 6). Those results suggest that PVZB3001 exhibits anti-tumor effect by inhibiting the enzymatic activity of IDO1 in vivo. Thus, PVZB3001 is expected to be a lead compound for a novel immunotherapeutic agent. However, since PVZB3001 couldn't completely suppress the growth of tumor, structural optimization is necessary to assess the potential for therapeutic application.

Recently, inhibition of Kyn pathway in tumor environment was reported to subsequently activate alternative NAD\textsuperscript{+} generation pathway in ovarian cancer and increase of NAD\textsuperscript{+} inhibit T cell proliferation and function via A2a/A2b purinergic receptor in mouse model.\textsuperscript{80} To improve antitumor efficacy of PVZB3001 treatment, analysis of NAD\textsuperscript{+} increase by alternative pathway and combination therapy of PVZB3001 and A2a/A2b antagonist in mice bearing CT26-Ido1 cells will be a subject for future study.

In conclusion, we identified MEK inhibitors and a bisisothiourea derivative as inhibitors of Kyn production in cells using the Kyn production assay with A172 cells. Pharmacological examination with MEK inhibitors and further knockdown analysis demonstrated the importance of the MEK/ERK pathway for the constitutive expression of TDO. On the other hand, the bisisothiourea derivative, PVZB3001 was found to be a selective and cell-permeable dual IDO1-TDO inhibitor. Those small molecules could be useful tools for modulating the Kyn pathway both in vitro and in vivo.

Authors Contributions
Conceptualization, S.Y., H.M., N.O. A.A.; methodology, S.Y., T.I., S.F., Y.K., K.O. H.M., D.M. N.O.; formal analysis, S.Y.; resources, K.O., N.O., A.A.; data curation, S.Y., T.I., S.F., Y.K., K.O., H.M., N.O., A.A.; investigation, S.Y., T.I., S.F., Y.K., K.O., H.M., N.O., D.M.; visualization, S.Y., N.O.; validation, S.Y., K.O.; writing—original draft preparation, S.Y.; writing—review and editing, K.O., H.M., N.O., D.M., O.T., A.A.; supervision, A.A.; project administration, A.A., funding acquisition, A.A. All authors have read and agree to the published version of the manuscript.

Data Availability
All data presented in this study are contained within the manuscript and the supporting information.

Supplemental Material
Supplemental material for this article is available online.

REFERENCES
1. Rafice SA, Chauhan N, Efimov I, Baran J, Raven EL. Oxidation of L-tryptophan in biology: a comparison between tryptophan 2,3-dioxygenase and indoleamine 2,3-dioxygenase. Biochem Soc Trans. 2009;37:408-412.
2. Théate I, van Baren N, Pilotte L, et al. Extensive profiling of the expression of the indoleamine 2,3-dioxygenase 1 protein in normal and tumor human tissues. Cancer Immunol Res. 2015;3:161-172.
3. Ball HJ, Sanchez-Perez A, Weiser S, et al. Characterization of an indoleamine 2,3-dioxygenase-like protein found in humans and mice. Gene. 2007;396:203-213.
4. Wu W, Nicolazzo JA, Wen L, et al. Expression of tryptophan 2,3-Dioxygenase and production of kynurenine pathway metabolites in triple transgenic mice and human Alzheimer's disease brain. PLoS One. 2013;8:e59749.
5. Cheong JE, Sun L. Targeting the IDO1/TDO2-KYN-AhR pathway for cancer immunotherapy - challenges and opportunities. Trends Pharmacol Sci. 2018;39:307-325.
6. Triplet TA, Garrison KC, Marshall N, et al. Reversal of indoleamine 2,3-dioxygenase-mediated cancer immune suppression by systemic kynurenic depletion with a therapeutic enzyme. Nat Biotechnol. 2018;36:758-764.
7. Hou DY, Muller AJ, Sharma MD, et al. Inhibition of indoleamine 2,3-dioxygenase in dendritic cells by stereoisomers of 1-methyl-tryptophan correlates with antitumor responses. Cancer Res. 2007;67:792-801.
8. Opitz CA, Litzenburger UM, Sahm F, et al. An endogenous tumour-promoting ligand of the human aryl hydrocarbon receptor. Nature. 2011;478:197-203.
9. Chon SY, Hassanain HH, Gupta SL. Cooperative role of interferon regulatory factor 1 and p91 (STAT1) response elements in interferon-gamma-inducible expression of human indoleamine 2,3-dioxygenase gene. J Biol Chem. 1996;271:17247-17252.
10. Takikawa O, Kuroiwa T, Yamazaki F, Kido R. Mechanism of interferon-gamma action. Characterization of indoleamine 2,3-dioxygenase in cultured human cells induced by interferon-gamma and evaluation of the enzyme-mediated tryptophan degradation in its anticytotoxic activity. J Biol Chem. 1998;263:2041-2048.
11. Litzenburger UM, Opitz CA, Sahm F, et al. Constitutive IDO expression in human cancer is sustained by an autocrine signaling loop involving IL-6, STAT3 and the AHR. Oncotarget. 2014;5:10838-1051.
12. van Baren N, Van den Eynde BJ. Tryptophan-degrading enzymes in tumoral immune resistance. Front Immunol. 2015;6:34.
13. Ye Z, Yue L, Shi J, Shao M, Wu T. Role of IDO and TDO in cancers and related diseases and the therapeutic implications. J Cancer. 2019;10:2771-2782.
14. Sumimoto M, Takahara K, Zennami K, et al. Tryptophan 2,3-dioxygenase in tumor cells is associated with resistance to immunotherapy in renal cell carcinoma. Cancer Sci. 2021;112:1038-1047.
15. Schramme F, Croisignani S, Frederik K, et al. Inhibition of tryptophan-dioxygenase activity increases the antitumor efficacy of immune checkpoint inhibitors. Cancer Immunol Res. 2020;8:32-45.
16. Badawy AA. Kynurenine pathway of tryptophan metabolism: Regulatory and functional aspects. Int J Tryptophan Res. 2017;10:117864917691938.
17. Salter M, Pogson C. The role of tryptophan 2,3-dioxygenase in the hormonal control of tryptophan metabolism in isolated rat liver cells: effects of glucocorticoids and experimental diabetes. Biochem J. 1985;229:499-504.

18. Le Naour J, Galluzzi L, Zitvogel L, Kroemer G, Vacchelli E. Trial watch: IDO inhibitors in cancer therapy. Oncol Immunol. 2020;9:1777625.

19. Naing A, Eder JP, Pha-Paul SA, et al. Preclinical investigations of a first-in-human phase 1 trial of M4112, the first dual inhibitor of indoleamine 2,3-dioxygenase and tryptophan 2,3-dioxygenase, in patients with advanced solid tumors. J Immunother Cancer. 2020;8:e000870.

20. Pei Z, Mendonca R, Gazzard L, et al. Aminoisoxazoles as potent inhibitors of tryptophan 2,3-dioxygenase 2 (TDO2). ACS Med Chem Lett. 2018;9:417-421.

21. Ning XL, Li YZ, Huo C, et al. X-ray structure-guided discovery of a potent, orally bioavailable, dual human indoleamine/tryptophan 2,3-Dioxygenase (hIDO/hTDO) inhibitor that shows activity in a mouse model of Parkinson’s Disease. J Med Chem. 2021;64:8303-8332.

22. Parr BT, Pastori R, Sellers BD, et al. Implementation of the CYP index for the design of selective tryptophan-2,3-dioxygenase inhibitors. ACS Med Chem Lett. 2020;11:541-549.

23. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2 ΔΔCT method. Methods. 2001;25:402-408.

24. Case DA, Cheatham TE 3rd, Darden T, et al. The Amber biomolecular simulation program. J Comput Chem. 2005;26:1668-1688.

25. Gerber PR, Müller K. MAB, a generally applicable molecular force field for structure modelling in medicinal chemistry. J Comput Aided Mol Des. 1995;9:255-268.

26. Soga S, Shirai H, Koborv M, Hirayama N. Use of amino acid composition to predict ligand-binding sites. J Chem Inf Model. 2007;47:400-406.

27. Corbeil CR, Williams CI, Labute P. Variability in docking success rates due to dataset preparation. J Comput Aided Mol Des. 2012;26:775-786.

28. Nakano S, Takai K, Isaka Y, et al. Identification of novel kynurenine production-inhibiting benzenesulfonamide derivatives in cancer cells. Biochem Biophys Res Commun. 2012;419:556-561.

29. Pilote L, Larrieu P, Stroobant V, et al. Reversal of tumoral immune resistance by inhibition of tryptophan 2,3-dioxygenase. Proc Natl Acad Sci U S A. 2010;107:2497-2502.

30. Salter M, Hazelwood R, Pogson C, Iyer R, Madge DJ. The effects of a novel and selective inhibitor of tryptophan 2,3-dioxygenase on tryptophan and serotonin metabolism in the rat. Biochem Pharmacol. 1995;49:1435-1442.

31. Matsumoto T, Aiki K, Isaka Y, et al. S-benzylisothiourea derivatives as small-molecular inhibitors of indoleamine 2,3-dioxygenase. Bioorg Med Chem Lett. 2010;20:5126-5129.

32. Cady SG, Sono M. 1-methyl-dl-tryptophan, beta-(3-benzofuranyl)-dl-alanine (the oxygen analog of tryptophan), and beta-[3-benzo[b]thienyl]-dl-alanine (the sulfur analog of tryptophan) are competitive inhibitors for indoleamine 2,3-dioxygenase. Arch Biochem Biophys. 1991;291:136-141.

33. Robin T, Revenu S, Urbakh M. Single-molecule theory of enzymatic inhibition. Nat Commun. 2018;9:779.

34. Du L, Xin Z, Tao B, et al. Both IDO1 and TDO contribute to the malignancy of peripheral blood mononuclear cells. Cell Biochem Funct. 2013;31:361-364.

35. Frumento G, Rondono R, Tonetti M, Diamante G, Benatti U, Ferrara GB. Tryptophan-derived catabolites are responsible for inhibition of T and natural killer cell proliferation induced by indoleamine 2,3-dioxygenase. J Exp Med. 2002;196:459-468.

36. Della Chiesa M, Carlomagno S, Frumento G, et al. The tryptophan catabolite L-kynurenine inhibits the expression of AKT and induces apoptosis in triple negative breast cancer cells. J Biol Chem. 2015;290:21644-21657.

37. Kochel TJ, Reader JC, Ma X, Kundu N, Fulton AM. Multiple drug resistance-associated protein (MRP) exports prostaglandin E2 (PGE2) and contributes to metastasis in basal/triple negative breast cancer. Int J Mol Sci. 2012;13:4351-4366.

38. Kochel TJ, Reader JC, Ma X, Kundu N, Fulton AM. Multiple drug resistance-associated protein (MRP) exports prostaglandin E2 (PGE2) and contributes to metastasis in basal/triple negative breast cancer. Int J Mol Sci. 2012;13:4351-4366.

39. Kochel TJ, Reader JC, Ma X, Kundu N, Fulton AM. Multiple drug resistance-associated protein (MRP) exports prostaglandin E2 (PGE2) and contributes to metastasis in basal/triple negative breast cancer. Int J Mol Sci. 2012;13:4351-4366.

40. Kochel TJ, Reader JC, Ma X, Kundu N, Fulton AM. Multiple drug resistance-associated protein (MRP) exports prostaglandin E2 (PGE2) and contributes to metastasis in basal/triple negative breast cancer. Int J Mol Sci. 2012;13:4351-4366.

41. Della Chiesa M, Carlomagno S, Frumento G, et al. The tryptophan catabolite L-kynurenine inhibits the expression of AKT and induces apoptosis in triple negative breast cancer cells. J Biol Chem. 2015;290:21644-21657.

42. Kochel TJ, Reader JC, Ma X, Kundu N, Fulton AM. Multiple drug resistance-associated protein (MRP) exports prostaglandin E2 (PGE2) and contributes to metastasis in basal/triple negative breast cancer. Int J Mol Sci. 2012;13:4351-4366.

43. Della Chiesa M, Carlomagno S, Frumento G, et al. The tryptophan catabolite L-kynurenine inhibits the expression of AKT and induces apoptosis in triple negative breast cancer cells. J Biol Chem. 2015;290:21644-21657.
71. Hoving S, Bar-Shimon M, Tijmes JJ, Godshleger R, Tal DM, Karlish SJ. Novel aromatic isothiouronium derivatives which act as high affinity competitive antagonists of alkali metal cations on Na/K-ATPase. *J Biol Chem*. 1995;270:29788-29793.

72. Jean-Jacques J, Chafeev C, Fonarev M, Fu J. Aromatic and Heteroaromatic Compounds Useful in Treating Iron Disorders. World Intellectual Property Organization; 2008.

73. Yu SP. Na+, K+ -ATPase: the new face of an old player in pathogenesis and apoptotic/hybrid cell death. *Biochem Pharmacol.* 2003;66:1601-1609.

74. Beaumont C, Delaunay J, Hetet G, Grandchamp B, De Montalembert M, Tchernev G. Two new human DMT1 gene mutations in a patient with microcytic anemia, low ferritinemia, and liver iron overload. *Blood*. 2006;107:4168-4170.

75. Opits CA, Somariabias Patterson LF, Mohapatra SR, et al. The therapeutic potential of targeting tryptophan catabolism in cancer. *Br J Cancer*. 2020;122:30-44.

76. Prendergast GC, Malachowski WP, DuHadaway JB, Muller AJ. Discovery of IDO1 inhibitors: from bench to bedside. *Cancer Res*. 2017;77:6795-6811.

77. Tang K, Wu YH, Song Y, Yu B. Indoleamine 2,3-dioxygenase 1 (IDO1) inhibitors in clinical trials for cancer immunotherapy. *J Hematol Oncol*. 2021;14:68.

78. Rohrig UF, Majjigapu SR, Vogel P, Zoete V, Michielin O. Challenges in the discovery of indoleamine 2,3-Dioxygenase 1 (IDO1) inhibitors. *J Med Chem*. 2015;58:9421-9437.

79. Efimov I, Basran J, Sun X, et al. The mechanism of substrate inhibition in human indoleamine 2,3-dioxygenase. *J Am Chem Soc*. 2012;134:3034-3041.

80. Odunsi K, Qian F, Lugade AA, et al. Metabolic adaptation of ovarian tumors in patients treated with an IDO1 inhibitor constrains antitumor immune responses. *Sci Transl Med*. 2022;14:636.