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Comparison study of different indoleamine-2,3 dioxygenase inhibitors from the perspective of pharmacodynamic effects

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Abstract

Introduction: Indoleamine 2,3-dioxygenase (IDO) was a potential tumor immunotherapy target. IDO inhibitors showed inconsistent results in clinical trials, but no preclinical comparative study was reported. The purpose of this study was to evaluate the differences of representative IDO inhibitors (PCC0208009, INCB024360, NLG919) from the pharmacological perspective. **Methods:** In vitro experiments included: inhibition effects on IDO activity in cell and enzyme-based assay, effects on IDO expression in HeLa cells, and enhancement of proliferation and activation of peripheral blood mononuclear cell (PBMC). In vivo experiments included: pharmacokinetics and tumor distribution in CT26-bearing mice, effects on Kyn/Trp and anti-tumor effect and immunological mechanism in CT26 and B16F10 tumor-bearing mice. **Results:** Compared with INCB024360 and NLG919, PCC0208009 effectively inhibited IDO activity at lower dose 2 nM and longer duration more than 72 h, had higher enhancements on PBMC proliferation and activation, and could inhibit the IDO expression in Hela cells. The pharmacokinetics characteristics of three IDO inhibitors were similar in CT26-bearing mice. In CT26 and B16F10 tumor-bearing mice, PCC0208009 and INCB024360 had similar effects in Kyn/Trp reduction, and more potent than NLG919; three IDO inhibitors had similar effects in tumor suppression, changes of the percentages of $CD3^+CD8^+$ and $CD3^+CD4^+$ T cells, and activation of tumor infiltrating lymphocytes, while PCC0208009 had a better tendency than INCB024360 and NLG919. **Conclusion:** PCC0208009, INCB024360, and NLG919 were all effective IDO inhibitors, but the comprehensive pharmacological activity of PCC0208009 was better than INCB024360 and NLG919, which was basically consistent with the results or progresses of clinical trials.

Keywords
Immunotherapy, INCB024360, Indoleamine 2,3-dioxygenase 1, NLG919, PCC0208009, Pharmacodynamic effects

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Introduction

Great progress had been made in cancer treatment in recent years, and cancer immunotherapy was one of the most anticipated areas.[1] Cancer immunotherapy could control and destroy cancer cells by activating the immune system of cancer patients. Currently, several immune checkpoint inhibitors have been on the market, and quickly become the standards of care for many cancer types. However, the clinical efficacy of these checkpoint inhibitors was still not satisfied in

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clinical, with disease response rate only 20% to 30% and effective in a specific patient subset.[2] Immune escape was one of the major biological mechanisms of tumorigenesis and metastasis, which was an important factor affecting the therapeutic effect of cancer treatment.[3,4]

Indoleamine-2,3 dioxygenase (IDO) was an enzyme converting tryptophan (Trp) into kynurenine (Kyn). IDO induced immune escape in the local tumor microenvironment through Trp depletion, Kyn accumulation, effector T-cells suppression, and regulatory T cells (Tregs) hyperactivation. IDO was highly expressed in a variety of tumor tissues, which was negatively correlated with patient survival rate.[5] Extensive research showed that IDO was a promising tumor immunotherapy target.[6] IDO inhibitors could reverse IDO-mediated immune tolerance, activate effector T cells, improve tumor microenvironment, and synergistically enhance the tumor killing inhibition of other drugs such as immunological checkpoint drugs and chemotherapy drugs. Development of IDO inhibitors was an important supplement to the clinical application of immunological checkpoint drugs.[7]

There were several IDO inhibitors in the clinical research stage, yet no drugs had been approved for marketing. Epacadostat (INCB024360), developed by Incyte Corporation, had provided surprising progression-free survival (PFS) and overall response rates (ORR) in multiple phase 1/2 single-arm trials. However, the confirmatory phase 3 study (ECHO-301) of Epacadostat did not meet its primary endpoint. At present, only proof-of-concept for non-small cell lung cancer in combination with pembrolizumab was ongoing in phase 2.[8] NLG-919 (navoximod), developed by NewLink Genetics, had been licensed to Roche. In 2017, Roche terminated the development of NLG-919 and return it back to NewLink, which was presumed that due to disappointing preliminary efficacy data. Linrodostat (BMS-986205), developed by Bristol-Myers Squibb (BMS), was the only IDO inhibitor in phase 3 clinical study at present. In 2018.10, a randomized, parallel-assigned, partially blinded, phase 3 trial (NCT03661320) was initiated to evaluate linrodostat in combination with neoadjuvant chemotherapy plus nivolumab in patients with muscle-invasive bladder cancer. PCC0208009 was Example 1 compound in the BMS patent "WO2015/031295 A1." Experiments in our laboratory have indicated that PCC0208009 was an effective IDO inhibitor, effectively inhibited tumor growth and prolong animal survival when in combination with temozolomide in animal glioma models.[9]

Different IDO inhibitors showed highly variable effects in clinical trials. The core structures of the above IDO inhibitors were different. INCB024360 was a hydroxyamidine compound, NLG919 was an imidazole compound, and PCC0208009 was a tetrazole compound. The differences in the core structure might determine the differential performances of different IDO inhibitors in clinical trials. However, it was unclear whether different IDO inhibitors had differences in the mechanisms of action, characteristics, or pharmacokinetics. Therefore, it was necessary to carry out comparative studies on the preclinical pharmacology and pharmacokinetics of these different types of IDO inhibitors. In this study, the differences of three representative IDO inhibitors, PCC0208009, INCB024360, and NLG919, were evaluated from the pharmacological perspective, which could provide a basis for the research and development of IDO inhibitors, especially the study of conformational relationships

Materials and methods

Cell lines, animals, and compounds

The human cervical cancer cell line HeLa was provided by Dr. Hongbo Wang (Yantai University) and cultured in Dulbecco's Modification of Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 50 IU/mL penicillin, and 50 μg/mL streptomycin sulfate. The mouse colon carcinoma cell line CT26 and mouse melanoma cell line B16F10 were obtained from Cell Bank of Chinese Academy of Sciences and cultured in RPMI 1640 supplemented with 10% FBS. The human peripheral blood mononuclear cells (PBMC) were purchased from Leide Biosciences and cultured in RPMI 1640 supplemented with 20% FBS. All cells were cultured at 37°C in a humidifide air atmosphere containing 5% CO_2.

Male C57BL/6 mice and BALB/c mice, 4–6 weeks, were provided by Beijing HFK Bioscience. The animal production license number was SCXK(Jing)2014-0004. All mice were quarantined and equilibrated to the new environment for 1 week and then maintain in a specific pathogen-free (SPF) environment. The animal

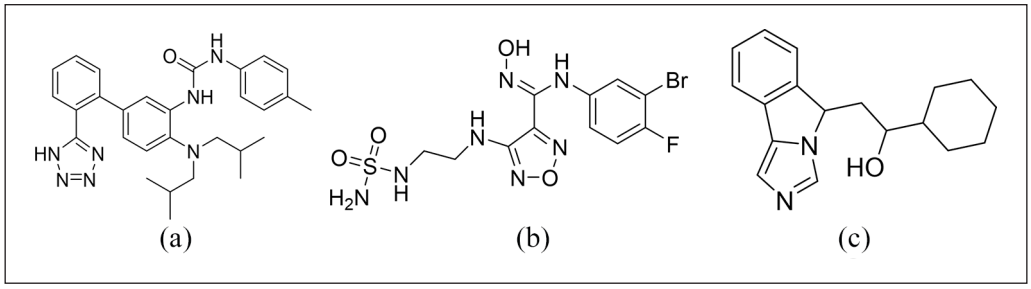

Figure 1. The chemical structure of compounds. (a) PCC0208009; (b) INCB024360; (c) NLG919.

rooms were maintained on a 12-h light/dark cycle at 21°C ± 5°C and 55% ± 15% relative humidity. Experiments related to animals required adherence to the guidelines for the Care and Use of Experimental Animal Research Committee in Yantai University (Approval number: YT-YX-2019-0097).

INCB024360 (CAS:1204669-58-8, $C_{11}H_{13}BrFN_7O_4S$) was purchased from Shanghai Send Pharmaceutical, with molecular weight of 438.2 and purity >98.5%. NLG919 (CAS: 1402836-58-1, $C_{18}H_{22}N_{20}$) was purchased from Hanxiang Biological Technology, with molecular weight of 282.4 and purity >98.7%. PCC0208009 was purchased from Shanghai Send Pharmaceutical, with molecular formula $C_{29}H_{35}N_7O$, molecular weight of 487.6, and purity >98.5%. The chemical structures of above three compounds were shown in Figure 1. For in vitro test, the compounds were dissolved in dimethyl sulfoxide (DMSO) and diluted to desired concentrations before use with DMEM or RPMI-1640. For in vivo tests, the compounds were dissolved in 20% Kolliphor® HS 15 (20% Solutol) to the desired concentrations.

Rabbit anti-human IDO antibody (Cat# 86630) was obtained from Cell Signaling Technology. PE-Cy7-conjugated anti-mouse CD3 mAb, PE-conjugated anti-mouse CD4 mAb and FITC-conjugated anti-mouse CD8 mAb (Cat# 558391) were provided by BD Biosciences. Mouse tumor dissociation kit (Cat# 130-097-730) was obtained from Miltenyi Biotech. Ficoll-Paque PREMIUM (Cat# 45-001-751) was obtained from GE Healthcare. Human interferon gamma (IFN-γ) ELISA kits (Catalog# SIF50), interleukin-2 (IL-2) ELISA kits (Cat# S2050), mouse IL-2 ELISA Kit (Catalog# SM2000), mouse IFN-γ ELISA Kit (Catalog# SMIF00), and human anti-CD3 mAb (Catalog# MAB100-500) were obtained from R&D Systems. L-Tryptophan (Cat# T8941), L-Kynurenine (Cat# K8625), Acetonitrile (Cat# 34851) were obtained from Sigma. 3-Nitro-L-tyrosine (Cat# N0905) was obtained from J&K. IDO1 enzyme (Cat# 71182) was obtained from BPS Bioscience.

IDO inhibition in cell-based and enzyme-based assay

In the HeLa cell-based assay, cells were seeded at 1.0×10^4 cells per well in 100 μL DMEM medium in 96-well plates. After culture 18 h, the medium was replaced with fresh medium containing 122 μM Trp, 122 μM Trp and 25 ng/mL IFN-γ, or 122 μM Trp and 25 ng/mL IFN-γ plus different IDO inhibitors with a maximum concentration of 5000 nM, 3-fold serial diluting to 0.3 nM, a total of 10 concentrations, three replicate wells per concentration. After incubated for 48 h, 50 μL of supernatant was transferred to a fresh 96-well plate, and 200 μL of acetonitrile was added. After centrifuged at 4000 rpm for 20 min at 4°C, 40 μL of supernatant was transferred to a fresh 96-well plate, and 360 μL of deionized water with 0.25 μg/mL of 3-Nitro-L-tyrosine were added, then the samples were detected with RapidFire/MS system (Agilent). The data was analyzed using GraphPad Prism 5.0. Fit the data with the model "log (Inhibitor) versus response-variable slope" and IC_{50} was calculated.

In the enzyme-based assay, different IDO inhibitors were dissolved and diluted with DMSO, and the highest concentration was 10 μM, 3-fold serial diluting to 0.2 nM, a total of 11 concentrations, three replicate wells per concentration. First, 25 μL of 120 nM IDO1 enzyme solution and 0.5 μL of compound solution were added into the reaction plate, mixed well and incubated for 30 min at room temperature. Then 25 μL of substrate (0.2 mM Trp) solution was added to each well. Centrifuged the plate at 1000 rpm for 1 min to mix and placed it on

the microplate reader SpectraMax M5e (MD). Set the temperature to 25°C, read OD_{320} every 10 min continuously for 60 min. The reaction systems containing only 0.2 mM Trp, or 0.2 mM Trp plus 120 nM IDO1 enzyme were set as a control. The data was analyzed using GraphPad Prism 5.0 and IC_{50} was calculated.

Dose and time dependence of inhibitory effects on IDO activity in HeLa cells

The model of HeLa cells with high IDO expression induced by IFN-γ was widely used for evaluation of IDO inhibitors. According to our previous experimental method,[9] cells were seeded at 4×10^3 cells/well in 96-well plates. After cultured overnight, the dose-response relationships and time-response relationships of three IDO inhibitors (INCB024360, NLG919, PCC0208009) on IDO activity were observed. In the dose-response relationship study, cells were treated with fresh medium (vehicle group), medium containing 100 ng/mL IFN-γ (IFN-γ group), or medium containing 100 ng/mL IFN-γ plus different IDO inhibitors with a maximum concentration of 5000 nM, 3-fold serial diluting to 0.3 nM, a total of 10 concentrations, three replicate wells per concentration. After incubated for 48 h, the culture supernatants were harvested. In the time-response relationship study, cells were treated with fresh medium (vehicle group), medium containing 100 ng/mL IFN-γ (IFN-γ group), or medium containing 100 ng/mL IFN-γ plus different IDO inhibitors at 10 nM or 100 nM. After incubated for 24 h, 48 h, and 72 h, the culture supernatants were harvested. All the samples were stored at –20°C for the determination of Kyn and Trp by liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS), and Kyn/Trp ratios were calculated based on the Kyn and Trp results.

Inhibition on IDO expression in HeLa cells

HeLa cells were seeded at 3×10^5 cells per well into 6-well plates. After cultured for 12 h, the medium was replaced with fresh medium, 100 ng/mL IFN-γ, or 100 ng/mL IFN-γ plus different IDO inhibitors at 200 nM. After incubated for 48 h, proteins were extracted from cells and the levels of IDO were analyzed by western blot as described previously.[10] The samples containing 50 μg protein were separated by 10% SDS-PAGE and transferred onto PVDF membrane. The membrane was blocked with 5% skimmed milk for 2 h at room temperature, and then incubated with anti-IDO antibody overnight at 4°C, followed by incubating with HRP-linked goat anti-rabbit IgG for 2 h. The membranes were examined by BeyoECL Plus reagent and expoused to the film. Protein expression levels were normalized by β-actin. This experiment was repeated three times. The optical density was quantified by Image-Pro Plus 6.0 software.

Proliferation and activation of PBMC in vitro

Intracellular IDO enzyme activity was increased when PBMC was activated.[11,12] So the proliferation and activiation of PBMC might be as a marker for IDO inhibitors. As described previously,[13] PBMC cells were seeded at 3×10^5 cells per well into 96-well plates. Cells were treated with RPMI 1640 medium (Vehicle control), 0.5 μg/mL of CD3 mAb, and 0.5 μg/mL CD3 mAb plus different IDO inhibitors at the concentrations of 1.6, 8, 40, 200, 1000 nM, respectively, three replicate wells per concentration. After incubated for 96 h, supernatants were collected for interleukin-2 (IL-2) and interferon-γ (IFN-γ) detection by ELISA kits. After the cell culture supernatants were collected, the cells were resuspended in RPMI1640 medium, and the number of PBMC cells was counted by microscopic counting method using a hemacytometer.

Pharmacokinetics and tumor distribution in tumor-bearing mice

Five-week-old male BALB/c mice were subcutaneously inoculated in the dorsal scapular with 0.2 mL of matrigel (BD Pharmingen) containing 5.0×10^5 CT26 tumor cells (1:1 volume matrigel/tumor cells) under ketamine-xylazine anesthesia. The length (a) and width (b) of xenografts were measured by digital calipers, and tumor volume (V) was calculated according to the formula: $V = 0.5 \times a \times b^2$. When the tumor volumes reached 300–400 mm^3, CT26 tumor-bearing mice were randomized into three groups: PCC0208009, INCB024360, NLG919. Animals were intragastrically (i.g.) administered with different IDO inhibitors at a single dose of 0.8 mmol/kg with volume of 0.1 mL/10 g. Before administration, and at 5, 30 min, 1, 2, 4, 8, 12 h after dosing, plasma and

tumors were collected for drug detection, each group containing four animals (n=4) per time point. The concentrations of different IDO inhibitors in plasma and tumor were determined using a validated LC-MS/MS method, and pharmacokinetic parameters were calculated by a non-compartmental model of WinNonlin Version 6.3 pharmacokinetic software.

Pharmacodynamic biomarker of Kyn/Trp in tumor-bearing mice

CT26-bearing mice were prepared as discribed in the section "2.6 Pharmacokinetics and tumor distribution in tumor-bearing mice." B16F10-bearing mice were prepared through a similar procedure. Five-week-old male C57/BL6 mice were subcutaneously inoculated in the dorsal scapular with 0.2 mL of matrigel containing 4.0×10^5 B16F10 cells. Both tumor-bearing mouse models were used in this study. When the tumor volumes reached 300–400 mm^3, mice were randomized into four groups: Vehicle, PCC0208009, INCB024360, and NLG919, each group containing five animals. Animals were i.g. administered with 20% solutol (vehicle) or different IDO inhibitors at 0.1, 0.3, 1.0 mmol/kg, twice daily for 7 days. At 2 h after the last dosing, plasma and tumors were collected for Kyn and Trp determination by the validated LC-MS/MS. Kyn/Trp ratios were calculated based on the Kyn and Trp results.

Anti-tumor effects in tumor-bearing mice

CT26-bearing mice and B16F10-bearing mice were used in the anti-tumor effects study of different IDO inhibitors. On the next day after the models were prepared, the tumor-bearing mice were randomized into four groups: Vehicle, PCC0208009, INCB024360, and NLG919, 10 animals in each group. Animals were i.g. administered twice daily with 20% solutol or different IDO inhibitors at 0.8 mmol/kg with volume of 0.1 mL/10 g.

Tumor volume and tumor weight. During the study, tumor volumes and body weights were measured once every 2 days. Tumor dynamic growth curve was made according to the tumor volumes at different time points. At the end of the study, tumors were collected and weighed, and the mean tumor weight (MTW) in each group was obtained, the inhibition rate (IR in %) was calculated using the formula: IR (%) = $[(MTW_{vehicle} - MTW_{treatment})/MTW_{vehicle}] \times 100$.

Flow cytometry for immunocyte analysis. At the end of the experiment, five tumors were randomly selected from each group for flow cytometry analysis. Tumor-infiltrating lymphocytes (TILs) were harvested from tumors by tumor dissociation kit. The suspensions that contain TILs were carefully added to the surface of Ficoll-Paque PREMIUM in a 15 mL centrifuge tubes. After centrifuged at 3000 rpm for 20 min, the cells at the interface were collected, then washed three times with DMEM containing 2% FBS and adjusted to a density of 10^7 cells/mL. TILs (10^6 cells/100 μL) were stained for 30 min with PE-Cy7-conjugated anti-mouse CD3, PE-conjugated anti-mouse CD4 antibodies, and FITC-conjugated anti-mouse CD8 antibody, then washing three times with PBS. Fluorescence-activated cell sorting (FACS) was performed with Accuri™ C6 flow cytometer, and the date were processed with C6 Flow Plus software.

Determination of IFN-γ and IL-2 levels. At the end of the experiment, the remaining five tumors in each group were used for the detection of IL-2 and IFN-γ secreted by TIL cells. TILs were normalized for CD3-positive cells by flow cytometry and cultured at a concentration of 1×10^5 cells/mL in DMEM with 25 μM 2-mercaptoethanol, 1 mM HEPES, 50 μg/mL streptomycin sulfate, 50 IU/mL penicillin, and 5% FBS. After 48 h, cell culture supernatants from TILs were collected for the detection of IL-2 and IFN-γ levels by ELISA kit.

Statistical analysis

The results are presented as the mean ± S.D. and data were analyzed by two-tailed unpaired Student's t-tests for paired groups or one-way ANOVA for three or more groups with the PASW Statistics 18.0 software package. $P < 0.05$ was considered statistically significant.

Results

IDO inhibition in cell-based and enzyme-based assay

In the HeLa cell-based assay, the averaged IC_{50} value of PCC0208009, INCB024360 and NLG919

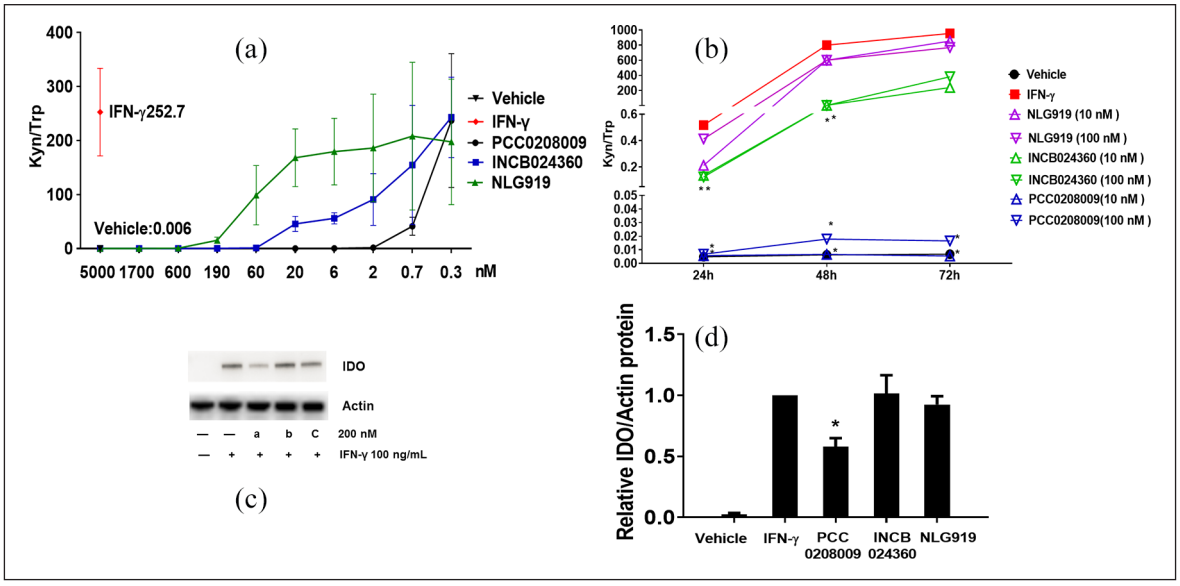

Figure 2. Inhibition effects of PCC0208009, INCB024360, and NLG919 on IDO activity and expression in HeLa cells. (a) Dose-response relationship study, cells were treated for 48 h with fresh medium, 100 ng/mL IFN-γ, or 100 ng/mL IFN-γ plus different IDO inhibitors with a maximum concentration of 5000 nM, 3-fold serial diluting to 0.3 nM, Kyn and Trp in the culture supernatants were determined by LC-MS/MS. (b) Time-response relationship study, cells were treated for 24 h, 48 h, and 72 h, with fresh medium, 100 ng/mL IFN-γ, or 100 ng/mL IFN-γ plus different IDO inhibitors at 10 nM or 100 nM, and Kyn and Trp in the culture supernatants were determined. (c, d) Effects on IDO expression, cells were treated for 48 h with fresh medium, 100 ng/mL IFN-γ, or 100 ng/mL IFN-γ plus different IDO inhibitors at 200 nM, the levels of IDO were analyzed by western blot (C), a, b, c was PCC0208009, INCB024360, and NLG919, respectively. Protein expression levels were normalized by using β-actin antibody. The optical density was quantified (D). *$P < 0.05$, compared with the IFN-γ group.

were 4.52 ± 1.19 nM, 12.22 ± 5.21 nM, 83.37 ± 9.59 nM, respectively. In the enzyme-based assay, the averaged IC_{50} value of INCB024360 and NLG919 were 35.23 ± 6.83 nM and 44.56 ± 7.17 nM, respectively. However, PCC0208009 did not show any activity in the enzyme-based assy. The above results indicated that the IDO inhibitory of PCC0208009 was better than INCB024360 and NLG919, and NLG919 had the lowest inhibitory activity.

Dose and time dependence of inhibitory effects on IDO activity in HeLa cells

In the dose-response relationship study, HeLa cells were treated with different IDO inhibitors at serial dilution concentrations for 48 h, and the results of Kyn/Trp in supernatants were shown in Figure 2(a). Kyn/Trp were effectively dose-dependently suppressed by PCC0208009, INCB024360, and NLG919 in the dose range of 0.3–5000 nM. When the Kyn/Trp was reduced from 252.7 (IFN-γ group) to about 1, the doses of PCC0208009, INCB024360, and NLG919 were 2 nM, 60 nM, and 600 nM, respectively. Therefore, PCC0208009 had more effectively inhibition on IDO activity than INCB024360 and NLG919.

In the time-response relationship study, cells were treated with different IDO inhibitors at 10 nM or 100 nM for 24 h, 48 h, and 72 h, and the results of Kyn/Trp were shown in Figure 2(b). At 10 nM and 100 nM dose levels, the ratios of Kyn/Trp in PCC0208009 groups were all significantly lower than that of IFN-γ group at 24 h, 48 h, and 72 h ($P < 0.05$). The ratios of Kyn/Trp in INCB024360 10 nM and 100 nM groups were significantly lower than that of IFN-γ group only at 24 h, and 24 h, and 48 h ($P < 0.05$), respectively. However, the ratios of Kyn/Trp in NLG919 group were significantly lower than that of IFN-γ group only at 24 h in 100 nM group ($P > 0.05$). The above results indicated that PCC0208009 was better than INCB024360 and NLG919, which could effectively inhibit the IDO activity at lower dose (10 nM), and last longer duration of inhibition, more than 72 h.

Inhibition on IDO expression in HeLa cells

HeLa cells were treated with IFN-γ plus different IDO inhibitors at 200 nM for 48 h, the protein

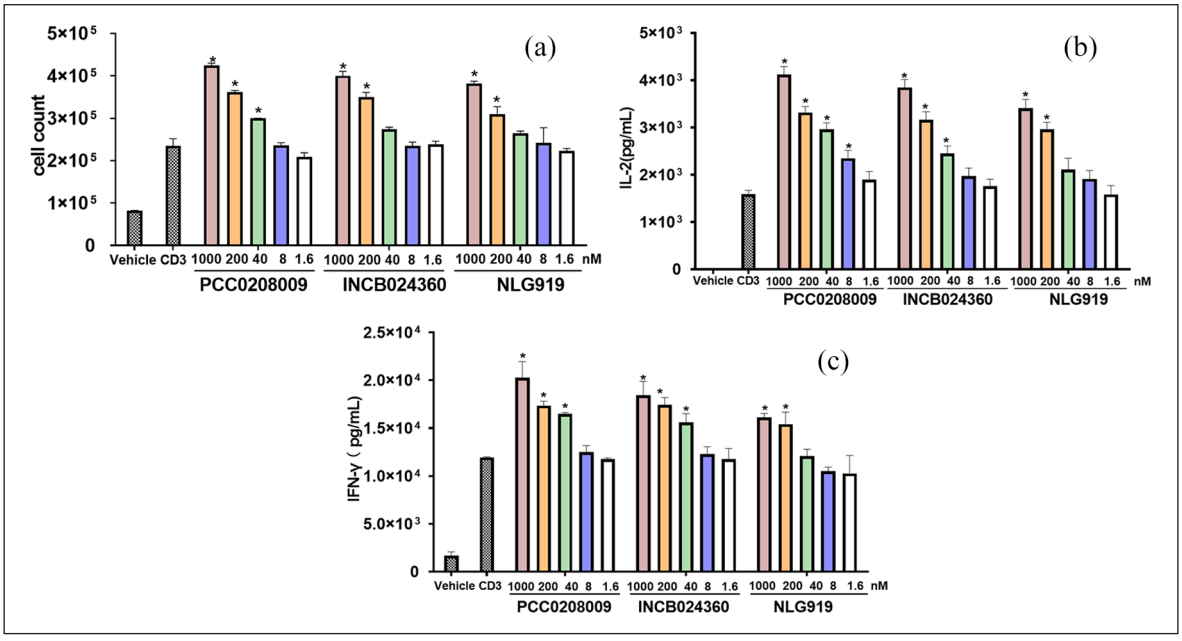

Figure 3. The ability of different IDO inhibitors to promote PBMC proliferation and cytokine secretion. PBMC cells were treated for 96 h with fresh growth medium (vehicle), 0.5 μg/mL of CD3 mAb, and 0.5 μg/mL CD3 mAb plus PCC0208009, INCB024360 or NLG919 at the concentrations of 1.6, 8, 40, 200, 1000 nM, respectively, IL-2 and IFN-γ levels in the supernatant were measurement by ELISA, and the number of PBMC cells was counted by a hemacytometer. *$P < 0.05$, compared with the CD3 mAb group.

expression levels of IDO were analyzed by western blot. The results were shown in Figure 2 (c and d) IDO expression in the vehicle group was almost undetectable, but significantly increased by IFN-γ induction. When PCC0208009 plus IFN-γ, the increase of IDO expression induced by IFN-γ were suppressed to some extentand, while INCB024360 and NLG919 had no such effects. This indicated that PCC0208009 had a different effect from INCB024360 and NLG919, inhibiting the expression of IDO in protein level.

Proliferation and activation of PBMC in vitro

The ability of different IDO inhibitors to promote PBMC proliferation and cytokine secretion were evaluated. PBMC were treated with CD3 mAb plus different IDO inhibitors at different concentrations for 96 h, the number of PBMC and the levels of IL-2 and IFN-γ were determined. As shown in Figure 3(a), CD3 mAb significantly stimulated cell proliferation; PCC0208009, INCB024360, and NLG919 further significantly increased the proliferation of PBMC at above 40 nM, 200 nM, and 200 nM, respectively, compared with CD3 mAb group ($P < 0.05$). As shown in Figure 3(b and c), CD3 mAb significantly stimulated the secretions of IL-2 and IFN-γ; PCC0208009, INCB024360, and NLG919 further significantly increased the IL-2 secretion at above 8 nM, 40 nM, and 200 nM, and increased the IFN-γ secretion at above 40 nM, 40 nM, and 200 nM, respectively, compared with CD3 mAb group ($P < 0.05$). These data indicated that the ability of PCC0208009 on PBMC proliferation and cytokine secretion were higher than that of INCB024360 and NLG919.

Pharmacokinetics and tumor distribution in tumor-bearing mice

The characteristics of PK and tumor tissue distribution of three IDO inhibitors were evaluated in CT26-bearing mice. After animals were i.g. administered with IDO inhibitors at the same single dose of 0.8 mmol/kg, the concentrations of IDO inhibitors in plasma and tumor was determined. The mean drug concentration-time curve of different IDO inhibitors in plasma and tumor were show in Figure 4, and main pharmacokinetic parameters were shown in Table 1. In plasma, the AUC and C_{max} of PCC0208009 were slightly higher than those of INCB024360 and NLG919, and the $t_{1/2}$ of INCB024360 was about twice that of PCC0208009 and NLG919. In tumor, the AUC and C_{max} of NLG919 were slightly higher than those of INCB024360 and PCC0208009, and the $t_{1/2}$ of

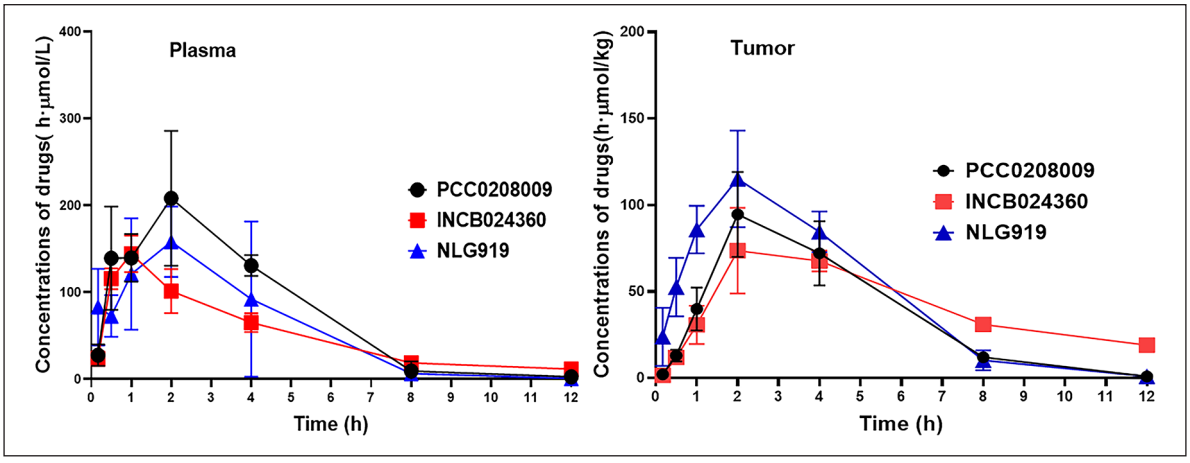

Figure 4. Mean drug concentration-time curve of different IDO inhibitors in plasma and tumor following i.g. administration (0.8 mmol/kg) in CT26-bearing mice (n = 4)

Table 1. Main PK parameters and tumor distribution of different IDO inhibitors following i.g. administration (0.8 mmol/kg) in CT26-bearing mice (n = 4).

Tissue	Drugs	AUC_{0-t} (h·μmol/L)	t_{max} (h)	C_{max} (μmol/L)	$t_{1/2}$ (h)
Plasma	PCC0208009	811.7	2	208.1	1.4
	INCB024360	583.6	1	144.2	3.1
	NLG919	603.9	2	158.1	1.2
Tissue	Drugs	AUC_{0-t} (h·μmol/kg)	t_{max}(h)	C_{max} (μmol/kg)	$t_{1/2}$ (h)
Tumor	PCC0208009	402.4	2	94.6	1
	INCB024360	492.5	2	73.7	4.4
	NLG919	505.3	2	115	1.3

INCB024360 was three to four times longer than that of PCC0208009 and NLG919. Considering the above results, the PK characteristics of three IDO inhibitors in this experiment were similar.

Effects on PD biomarker of Kyn/Trp in tumor-bearing mice

Kyn/Trp was the sensitive PD biomarker of IDO inhibitors.[14] B16F10 and CT26 bearing mice were i.g. administered with different IDO inhibitors at 0.1, 0.3, 1.0 mmol/kg, twice daily for 7 days. Kyn/Trp in plasma and tumors were determined at 2 h after the last dosing, which were shown in Figure 5. In CT26-bearing mice, Kyn/Trp in plasma and tumor were all dose-dependently suppressed by three drugs, which were significantly lower than that of the vehicle group ($P < 0.05$). In the high dose group (1.0 mmol/kg), inhibition rate of Kyn/Trp of PCC0208009, INCB024360, NLG919 were 91.7%, 87.6%, 74.7% in the plasma, and 91.0%, 88.7%, 73.8% in the tumor, respectively. In B16F10-bearing mice, Kyn/Trp in plasma and tumor were all dose-dependently suppressed by three drugs, which were significantly lower than that of the vehicle group ($P < 0.05$). In the high dose group (1.0 mmol/kg), inhibition rate of Kyn/Trp of PCC0208009, INCB024360, NLG919 were 91.8%, 85.0%, 72.5% in the plasma, and 89.5%, 81.1%, 75.9% in the tumor, respectively. These data indicated that three IDO inhibitors could all effectively reduce the Kyn/Trp in plasma and tumor in vivo. PCC0208009 and INCB024360 were similar in efficacy, and more potent than NLG919.

Anti-tumor effects in tumor-bearing mice

To compare the in vivo anti-tumor effects and mechanisms of different IDO inhibitors, CT26-bearing mice and B16F10-bearing mice were prepared separately. The tumor-bearing mice were i.g. administered with 20% solutol or PCC0208009, INCB024360, and NLG919 at 0.8 mmol/kg, twice daily until the end of the study. Animal body

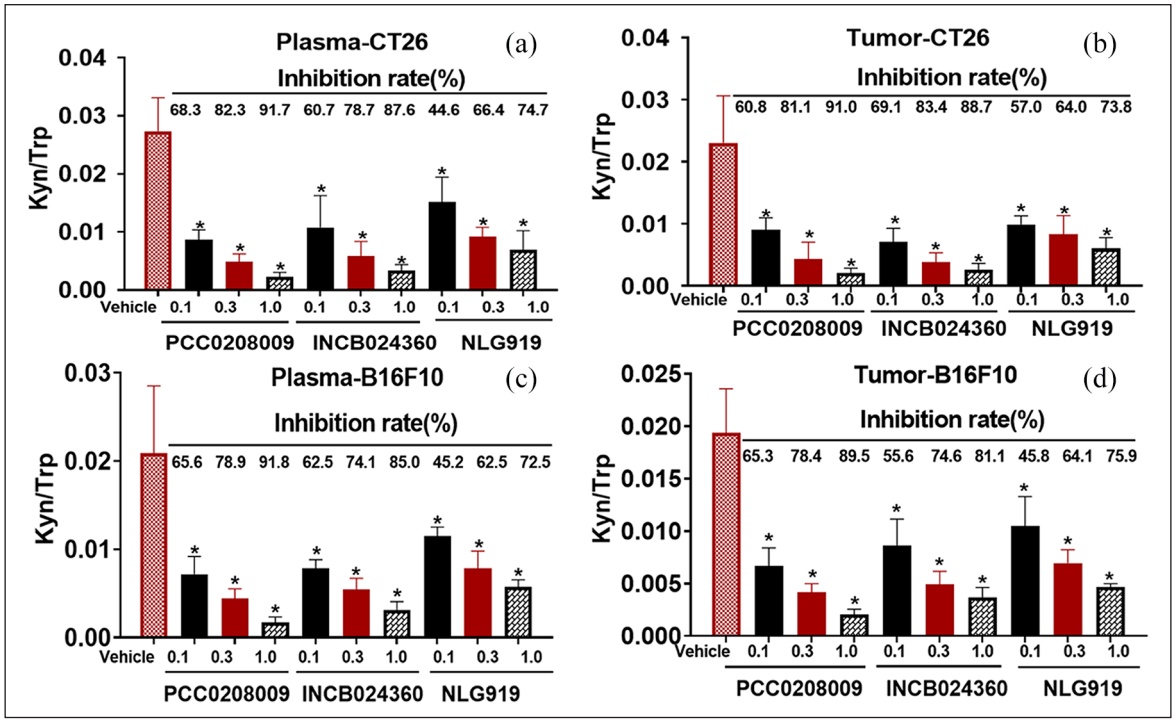

Figure 5. Effects on Kyn/Trp of different IDO inhibitors in CT26 and B16F10-bearing mice. CT26-bearing mice and B16F10-bearing mice were randomized into four groups: Vehicle, PCC0208009, INCB024360 and NLG919. Animals were i.g. administered with 20% solutol or PCC0208009, INCB024360, NLG919 at 0.1, 0.3, 1.0 mmol/kg, respectively, twice daily for 7 days. At 2 h after the last dosing, plasma and tumors were collected for Kyn and Trp determination by LC-MS/MS, and Kyn/Trp ratios were calculated. n = 5, *$P < 0.05$, compared with the Vehicle group.

Table 2. Effects of PCC0208009, INCB024360, and NLG919 on body weights and tumor weights.

	Groups	Body weight (g)			Tumor weight (g)	Inhibition rate (%)
		Pre-treatment (day 1)	Post-treatment (day17)	Weight gain (%)		
CT26	Vehicle	21.39 ± 1.04	22.49 ± 1.29	5.14%	2.46 ± 0.42	—
	PCC0208009	20.65 ± 1.31	20.99 ± 1.02	1.64%	1.54 ± 0.31*	37.4%
	INCB024360	20.14 ± 0.91	19.90 ± 0.67	−1.19%	1.61 ± 0.25*	34.6%
	NLG919	21.28 ± 1.18	20.98 ± 0.78	−1.40%	1.87 ± 0.28	23.9%
B16F10	Vehicle	21.08 ± 1.06	21.99 ± 1.16	4.32%	2.81 ± 0.59	—
	PCC0208009	21.00 ± 1.31	21.67 ± 0.48	3.19%	1.69 ± 0.36*	39.7%
	INCB024360	22.05 ± 1.34	21.75 ± 1.46	−1.36%	1.90 ± 0.55*	32.3%
	NLG919	21.06 ± 0.96	20.83 ± 0.68	−1.09%	1.95 ± 0.46*	30.6%

CT26-bearing mice and B16F10-bearing mice were i.g. administered twice daily with 20% solutol or different IDO inhibitors at 0.8 mmol/kg (n = 10). During the study, the body weight of animals and tumor volumes were measured once every 2 days and the percentage of body weight gain was calculated based on that at the beginning of study. At the end of the study, tumors were collected and weighed, and the inhibition rates were calculated. *$P < 0.05$, compared with the vehicle group.

weights, tumor volume and weights, T cell populations in tumor, and IFN-γ and IL-2 secretion were observed or measured.

Body weight of tumor-bearing mice. As shown in Table 2 and Figure 6(a and b), PCC0208009, INCB024360 and NLG919 at 0.8 mmol/kg had no significant effects on the body weights of animals in both CT26 and B16F10 models.

Tumor volume and tumor weight. The tumor growth curves of CT26 and B16F10 were shown in Figure 6(c and d). In both models, the suppressions of tumor growth were observed in all IDO inhibitor

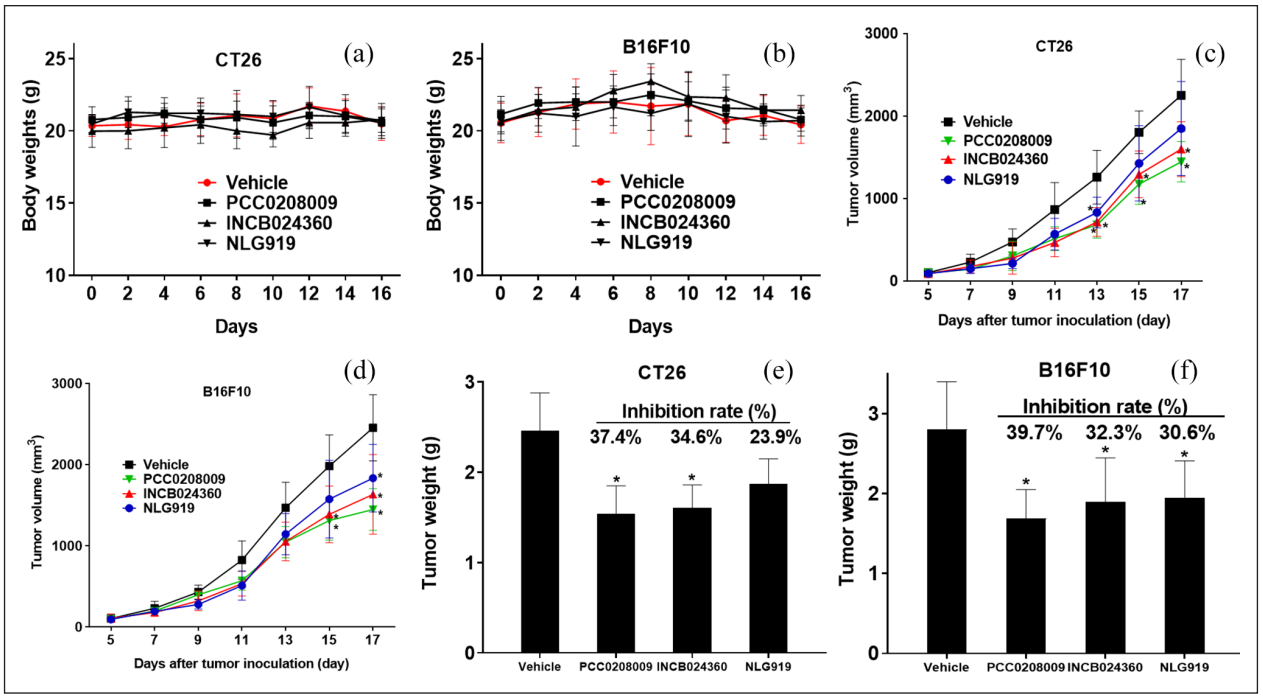

Figure 6. Anti-tumor effects of different IDO inhibitors on CT26 and B16F10 bearing mice. Tumor-bearing mice were randomized into four groups: Vehicle, PCC0208009, INCB024360, NLG919. Animals were i.g. administered twice daily with 20% solutol or different IDO inhibitors at 0.8 mmol/kg. The body weight of animals (a, b) and tumor volumes were measured once every 2 days. Tumor dynamic growth curve was made according to the tumor volumes at different time points (c, d). At the end of the study, tumors were collected and weighed, and the inhibition rate (IR in %) of mean tumor weight were calculated (e, f). n = 10, $^*P < 0.05$, compared with the vehicle group.

groups. In CT26-bearing mouse model, the mean tumor volumes of PCC0208009, INCB024360 and NLG919 groups were significantly smaller than that of the vehicle group on days 13–17, days 13–17, and day 13, respectively ($P < 0.05$). In B16F10-bearing mouse model, the mean tumor volumes of PCC0208009, INCB024360, and NLG919 groups were significantly smaller than that of the vehicle group on days 15–17, days 15–17, and day 17, respectively ($P < 0.05$). However, there were no significant differences when compared between groups of different IDO inhibitors ($P > 0.05$).

At the end of the experiment, the mean tumor weights and inhibition rates were observed (Figure 6(e and f), Table 2). In CT26-bearing mouse model, the mean tumor weights in PCC0208009 and INCB024360 groups were significantly smaller than those in the vehicle group ($P < 0.05$); The mean tumor weights in NLG919 group was smaller than that in the vehicle group, but no significantly difference ($P = 0.052$). The inhibition rates of PCC0208009, INCB024360, and NLG919 groups were 37.4%, 34.6%, and 23.9%, respectively. In B16F10-bearing mouse model, the mean tumor weights in PCC0208009, INCB024360, and NLG919 groups were significantly smaller than those in the vehicle group ($P < 0.05$), with tumor inhibition rates were 39.7%, 32.3%, and 30.6%, respectively. In both models, there were no significant differences when compared between groups of different IDO inhibitors ($P > 0.05$).

Flow cytometry for immunocyte analysis. In order to compare the three IDO inhibitors from the immunological mechanism, T cell populations within tumors were analyzed. As shown in Figure 7(a and b), compared with the vehicle group, the percentages of $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells in the PCC0208009, INCB024360, and NLG919 groups were slightly increased, ranging from 18% to 44% in CT26 model and 20%–47% in B16F10 model, but there were no significant differences ($P > 0.05$). There was also no significant difference between IDO inhibitor groups ($P > 0.05$).

Determination of IFN-γ and IL-2 levels. The effects of different IDO inhibitors on the functions of

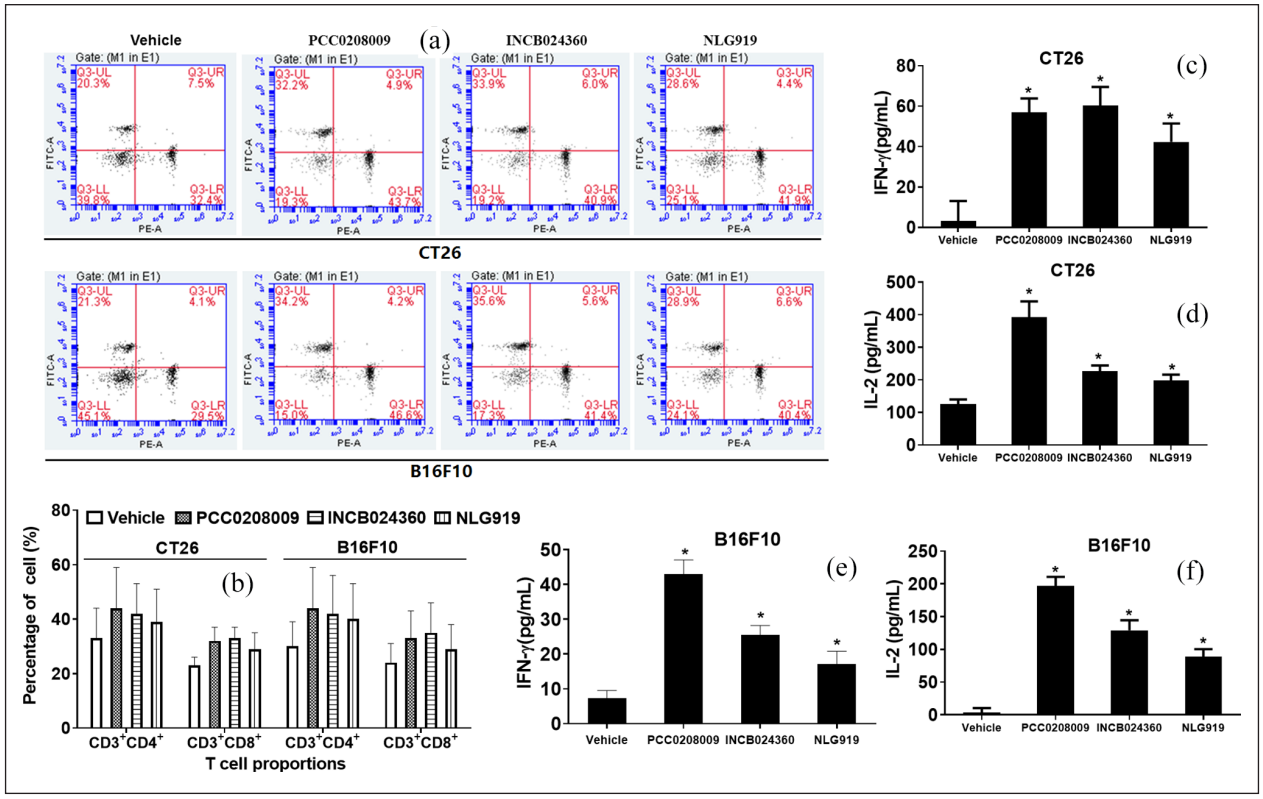

Figure 7. Effects of different IDO inhibitors on subtypes and functions of tumor infiltrating T cells. CT26-bearing mice and B16F10-bearing mice were twice daily i.g. administered with vehicle (20% solutol) or PCC0208009, INCB024360, and NLG919 at 0.8 mmol/kg, respectively. At the end of the study, five tumors were randomly selected from each group for flow cytometry with PE-Cy7-CD3e, PE-CD4, and FITC-CD8. The remaining five tumors in each group were used for the detection of IFN-γ and IL-2. TILs were normalized and cultured for 48 h, and the IFN-γ and IL-2 levels in the supernatants were determined by ELISA kit. *$P < 0.05$, compared with the vehicle group.

lymphocytes infiltrated in B16F10 and CT26 tumors were investigated by measuring the contants of IFN-γ and IL-2. The results from CT26 model were shown in Figure 7(c and d), the concentrations of IL-2 secreted by TILs from PCC0208009, INCB024360, and NLG919 groups were 3.1-fold, 1.8-fold, and 1.6-fold higher than that from the vehicle group ($P < 0.05$), respectively. The concentrations of IFN-γ from PCC0208009, INCB024360, and NLG919 groups were also significantly higher than that from the vehicle group ($P < 0.05$), with 4.5-fold, 3.9-fold, and 3.4-fold, respectively. The levels of both IFN-γ and IL-2 between three IDO inhibitors exhibited no significant difference ($P > 0.05$), however, PCC0208009 has a better tendency than INCB024360 and NLG919 in promoting the secretions of IFN-γ and IL-2. In the B16F10 model, the results were similar, which were shown in Figure 7(e and f). The concentrations of IL-2 and IFN-γ in the groups of IDO inhibitors were significantly higher than that in the vehicle group ($P < 0.05$), with 4.4-fold, 2.9-fold, 2.0-fold in IL-2, and 5.8-fold, 3.5-fold, 2.0-fold in IFN-γ, respectively.

Discussion

The listed immunological checkpoint drugs up-to-date have shown low clinical response rate, which may be associated with their co-expression with IDO or induction of IDO expression.[15] The results of data from pre-clinical and early clinical trials also have shown that IDO inhibitors could effectively enhance the efficacy of PD-1 and other immune checkpoint drugs.[16] The development of IDO inhibitors was significant addition to the clinical application of immunological checkpoint drugs.[7] However, the first phase 3 trial (ECHO-301) to evaluate INCB024360 (epacadostat) in combination with pembrolizumab in advanced melanoma did not meet its primary endpoints.[17] This

failure led to the decision of the three companies to suspend, cancel or reduce 13 trials of IDO1 inhibitors in combination with immune checkpoint inhibitors.[15] There were several possible reasons for the failure of ECHO-301: No screening for biomarkers suitable for IDO inhibitor; Epacadostat was a selective IDO inhibitor and had weak therapeutic effects for patients with TDO expression; The dose (100 mg BID) in this trial might not be the best dose.[18] Therefore, better rationalized compounds and trial designs will be important in the future.

The effects of different IDO inhibitors in clinical trials varied greatly. Currently, proof-of-concept for NSCLC of INCB024360 was ongoing in clinical phase 2 after the failure of ECHO-301; NLG-919 was terminated in phase 1 clinical trial; Only BMS-986205 was in phase 3 clinical study, plus nivolumab in bladder cancer patients. Different IDO inhibitors showed inconsistent results in clinical trials. No pre-clinical studies on the comparison of these different IDO inhibitors were reported. Therefore, in this study, three representative IDO inhibitors were compared from pharmacodynamics and pharmacokinetics perspective.

In the HeLa cell-based assay, PCC0208009 had the lowest IC_{50} value with 4.52 ± 1.19 nM, and effectively inhibited the IDO activity at lower dose 2 nM and longer duration more than 72 h, which were all better than INCB024360 and NLG919. However, it was important to mention that PCC0208009 was not active in the enzyme-based assay and inhibited the protein expression of IDO in HeLa cell, which were significantly different from INCB024360 and NLG919. IDO inhibition is expected to work by impacting the activity of lymphocytes. Th1 cells mainly secrete cytokines such as IL-2 and IFN-γ are the main cytokines expressed by activated T cells, and contribute to the induction of the tumor antigen-specific Th1 immune response in cancer models. So, in this study, IFN-γ and IL-2 were chosen for evaluation of lymphocyte function. PCC0208009 showed stronger activity to promote the proliferation and IL-2 and IFN-γ secretion of PBMC than that of INCB024360 and NLG919. The results of the above studies indicated that PCC0208009 had better pharmacological activities in vitro and the mechanism of action of PCC0208009 was different from INCB024360 and NLG919.

In the PK and tumor tissue distribution study of CT26-bearing mice, PCC0208009, INCB024360, and NLG919 had the similar PK characteristics, with equivalent AUC and C_{max} in plasma and tumor. This means that the differences in the in vivo activity of these three IDO inhibitors may mainly depend on their own inhibitory activity on IDO. In the B16F10 and CT26 bearing mice, PCC0208009, INCB024360, and NLG919 could all effectively reduce the Kyn/Trp, a sensitive PD biomarker in plasma and tumor. PCC0208009 and INCB024360 were similar in efficacy, and more potent than NLG919.

In the anti-tumor studies, CT26 and B16F10 tumor-bearing mice were i.g. administered with three IDO inhibitors at 0.8 mmol/kg, which was a dose close to maximum efficacy in the preliminary experiments in our laboratory. In both models, PCC0208009, INCB024360 and NLG919 significantly suppressed the tumor growths, and no significant differences between different IDO inhibitors. However, the anti-tumor effects of PCC0208009, INCB024360, and NLG919 decreased sequentially, only from the value of inhibition rate. Further, PCC0208009, INCB024360, and NLG919 increased the percentages of $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells in tumors, and promoted the activation and secretion of IFN-γ and IL-2 of tumor infiltrating lymphocytes. These changes had no significant difference between IDO inhibitors, while PCC0208009 has a better tendency than INCB024360 and NLG919.

This study may have some limitations. The first one could be related to the relatively small number of the samples in some experiments. The secend one was that the specific mechanism of PCC0208009 exerting IDO inhibitory effect was unclear. PCC0208009 had regulation effects on IDO expression, significantly different from the other two inhibitors. IDO protein is induced upon IFN-γ treatment in cells. Reduction in IDO expression by PCC0208009 may result from alteration of IFN-γ signaling or other mechanisms related to transcriptional regulation. However, our previous research results indicated PCC0208009 was still an IDO inhibitor.[9] Because at 0.5 h, PCC0208009 could significantly inhibit kyn/Trp, this is more like its direct inhibition on IDO. So, we speculated that PCC0208009 had two ways of functioning: one was directly inhibition on the IDO activity, the other was the secondary effects through regulation of IDO expression.

Conclusion

In summary, our findings indicated that PCC0208009, INCB024360, and NLG919 were all

effective IDO inhibitors, but the comprehensive pharmacological activity of PCC0208009 was better than that of INCB024360 and NLG919, which was basically consistent with the results or progress of clinical research.

Animal welfare

The present study followed international, national, and/or institutional guidelines for humane animal treatment and complied with relevant legislation.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethics approval

Ethical approval for this study was obtained from the Care and Use of Experimental Animal Research Committee in Yantai University (APPROVAL NUMBER: YT-YX-2019-0097).

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