Original Research

A combination of the metabolic enzyme inhibitor APO866 and the immune adjuvant L-1-methyl tryptophan induces additive antitumor activity

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Abstract

Many types of malignant cells have a higher nicotinamide adenine dinucleotide (NAD) turnover rate than normal cells, as well as the ability to escape immune responses. Indoleamine 2,3-dioxygenase (IDO) is reported to be a negative immune regulator. Overexpression of IDO in dendritic cells is observed in tumor-draining lymph nodes. IDO-expressing dendritic cells suppress T-cell activation and promote immune tolerance. The nicotinamide phosphoribosyl transferase (NAMPT) inhibitor APO866 (also called FK866 or WK175) selectively inhibits tumor growth through intracellular NAD depletion. The IDO-specific inhibitor L-1-methyl-tryptophan (L-1MT) activates immune responses and reduces tumor volume in murine tumor models. We combined L-1MT and APO866 treatments and tested their antitumor effects in the murine gastric and bladder tumor models. In immune-competent mice, a combination of APO866 and L-1MT had a better therapeutic effect than did either L-1MT or APO866 alone. The intracellular level of NAD was suppressed by APO866 but not L-1MT. However, an additive inhibitory effect on tumor growth was not observed in tumor-bearing immune-deficient mice. The new strategy of combining a metabolic inhibitor and an immune adjuvant induced a potent therapeutic effect.

Keywords: indoleamine 2,3-dioxygenase, 1-methyl tryptophan, APO866 (FK866, WK175), cancer therapy, combination therapy, gastric cancer, bladder cancer

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Introduction

Nicotinamide adenine dinucleotide (NAD) is a substrate for several biological enzymes including sirtuins and poly-(ADP-ribose) polymerases (PARPs), which are involved in aging, genomic stability and metabolism.[1–3] Many types of cancer cells have a higher turnover rate of NAD than normal cells because of genome instability and PARP1-dependent DNA repairs.[4] Tryptophan, nicotinic acid and nicotinamide are three major sources of NAD biosynthesis in mammals, and nicotinamide phosphoribosyl transferase (NAMPT) is the rate-limiting enzyme in the nicotinamide pathway.[5,6] APO866 (also called FK866 or WK175) is a specific inhibitor of NAMPT. Previous studies have indicated that APO866 exerts an antitumor and antiangiogenesis effect against several types of solid tumors and leukemias.[7–10]

Indoleamine 2,3-dioxygenase (IDO), the rate-limiting enzyme of the tryptophan degradation pathway, converts L-tryptophan to L-kynurenine.[11] The protein expression and enzyme activity of IDO are induced by interferon-γ in some types of cells, including tumor cells.[12] The function of IDO is linked to tumoral immune tolerance. Many types of human tumors constitutively express IDO, including tumors of gastric and bladder cancers.[13] On the other hand, IDO-expressing dendritic cells suppress T-cell activation and promote the proliferation of regulatory T cells

in tumor-draining lymph nodes in murine tumor models.[14,15] Moreover, IDO-expressing dendritic cells suppress proliferation of $CD4^+$ and $CD8^+$ T cells, and they increase the number of regulatory T cells.[16] Inhibition of IDO enzyme activity reverses immune suppression and enhances antitumor effect. One IDO inhibitor, 1-methyl tryptophan (1MT), has the ability to reduce the volume of tumors in immune-competent mice.[13,17] A combination of 1MT and a chemotherapy drug lead to rapid tumor regression.[18,19] This evidence indicates that suppression of IDO enzyme activity could induce an antitumor effect.

In recent studies, several anticancer agents were reported to induce cell death through the manipulation of cellular energy uptake, including the depletion of NAD.[20,21] However, the function of NAMPT is also linked to the inflammation and development of immune cells.[22,23] These reports implied that immune cells are suppressed by APO866. We hypothesized that a combination of metabolic enzyme inhibitor and immune adjuvant would induce an additive therapeutic effect. Meanwhile, IDO is also a metabolic enzyme of tryptophan, which is a precursor of NAD biosynthesis. Thus, inhibition of IDO activity in astroglioma cells with a competitive IDO inhibitor might suppress the production of NAD.[24] It will also be important to determine the intracellular level of NAD during the combination therapy.

IDO enzyme activity in tumor cells and dendritic cells was inhibited by 1MT.[12,25] The L-isoform of 1MT more preferentially inhibited IDO enzyme activity than the D-isoform.[19] Therefore, we studied the therapeutic effects of combining APO866 and L-1MT in the animal gastric and bladder tumor models. Our results indicate that the therapeutic effect was enhanced with a combination of APO866 and L-1MT. However, the level of intracellular NAD was not decreased by the addition of L-1MT *in vitro*. These findings suggest that the strategy of combining an IDO inhibitor with an NAMPT inhibitor could be employed in future clinical trials.

Materials and methods

Cell lines and animals

The murine hepatoma ML-1 cell, the murine colon cancer CT-26 cell and the murine bladder cancer MBT-2 cell have been described previously.[17,26] The murine gastric carcinoma cell line, MGCC3I, was established by Dr Yi-Ling Chen (s5887110@nckualumni.org.tw, Department of Senior Citizen Service Management, Chia-Nan University of Pharmacy and Science, 60, Erh-Jen Rd., Sec. 1, Jen-Te, Tainan 717, Taiwan (R.O.C.), manuscript submitted) and cultured in high glucose Dulbecco's modified Eagle's medium with 10% fetal bovine serum (Invitrogen Corp., Carlsbad, CA, USA) and 1% penicillin/streptomycin (Invitrogen Corp.). C3H/HeN mice, ICR mice and NOD.CB17-PRKDC (non-obese diabetes/severe combined immunodeficiency [NOD/SCID]) mice were obtained from the Laboratory Animal Center at National Cheng Kung University. All of the experiments were performed in six- to eight-week-old female mice. All of the mouse studies were approved by the Animal Welfare Committee at National Cheng Kung University.

Reagents

APO866 (FK866) was kindly provided by the National Institute for Mental Health, Chemical Synthesis and Drug Supply Program, Dr Ken Rehder (krehder@rti.org, 6001 Executive Boulevard, Bethesda, MD, USA), and dissolved in a dimethyl sulfoxide /saline mixed solvent at a 1:3 ratio and then stored at -20°C. A concentration of 25 mg/kg of APO866 was injected intraperitoneally into mice at a weekly interval. Interferon-γ was obtained from R&D systems (Minneapolis, MN, USA).

Western blot

Twenty-five micrograms of cell lysates were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred on to polyvinylidene fluoride membranes (Amersham Biosciences, Piscataway, NJ, USA). The antibodies for Western blotting were anti-mouse IDO (AdipoGen Inc., Incheon, South Korea), anti-NAMPT (Bethyl Laboratory Inc., Montgomery, TX, USA) and anti-β-actin (Chemicon, Temecula, CA, USA). Chemiluminescent horseradish peroxidase (HRP) substrate (Millipore, Billerica, MA, USA) was used for detection. The images of Western blots were obtained with the UVP BioSpectrum AC Imaging system using the UVP Vision Works software (Upland, CA, USA).

Measurement of IDO enzyme activity

Two hundred micrograms of the extracted cell lysate was used for the assay of IDO activity, following the protocol described previously.[17] Briefly, the cells were treated with 200 U of interferon-γ and the cell lysates were isolated for measurement of IDO activity.

Intracellular NAD concentration

MBT-2 and MGCC3I cells at a concentration of 3×10^5 and 2×10^5, respectively, were seeded into a six-well plate, and were then incubated in media containing APO866 (5 nmol/L) or L-1MT (500 μmol/L) for 24 h. The intracellular level of NAD and NADH was determined with an NAD/NADH quantification kit (Biovision, Mountain View, CA, USA) according to the manufacturer's instructions.

Cell proliferation assay

MBT-2 and MGCC3I cells at a concentration of 1×10^4 were seeded into 100 μL growth media in a 24-well plate. Twenty microliters of the Cell Titer 96 cell proliferation assay (methanethiosulfonate) reagents (Promega, Madison, WI, USA) were added to each well and incubated at 37°C for two hours at each time point. The absorbance was determined with an enzyme-linked immunosorbent assay reader (DYNATECH MR5000) at 490 nm.

Therapeutic efficacy on established tumor

The mice were injected subcutaneously in the flank region with 1×10^6 MBT-2 or MGCC3I cells in 0.5 mL of

phosphate-buffered saline. Seven days later, the mice were randomly assigned to each group. L-1MT (Sigma-Aldrich, Saint Louis, MO, USA) was dissolved in sterile water (5 mg/mL) and adjusted to pH 9.9 with NaOH. The mice were fed with L-1MT-supplemented water, which was replaced every two to three days. L-1MT is stable in pH 9.9 water for at least four days. The concentration of L-1MT was determined by high-performance liquid chromatography with a reverse-phase column (Mightysil RP-18 GP 4.6 250 mm; Kanto Chemical Co, Inc, Tokyo, Japan) and a mobile phase of 15 mmol/L sodium acetate–acetic acid solution containing 2.7% (v/v) acetonitrile (pH 3.6) at a flow rate of 1.0 mL/min. The control mice were fed with water at pH 9.9. The tumor size was measured using a caliper three times a week. The tumor volume was calculated using the formula: volume $= (A^2 \times B \times 0.5236)$, where A and B represent the shortest and longest diameter, respectively. The mice were sacrificed by cervical dislocation when the tumor volume exceeded 3500 mm^3.

Histological analysis

One week after the second APO866 injection, or two weeks after the L-1MT treatment (day 21 after tumor cell inoculation), the mice were sacrificed and the tumors were immersed in the optimal cutting temperature compound. Immunohistochemistry was performed on 5 μm cryostat sections. The antibodies used for detection included anti-CD4 (BD Pharmingen, San Diego, CA, USA), anti-CD8a (BD Pharmingen) and anti-CD31 (BD Pharmingen). Protein detection was performed with the mouse/rabbit PolyDetector HRP/DAB detection system (BioSB, Santa Barbara, CA, USA) according to the manufacturer's recommendation, and then counterstained with hematoxylin. Infiltrated T-cells or blood vessels were counted in five random fields in each sample, and then three samples from the independent experiments were counted.

Graph and statistics

All of the numerical data and graphs were analyzed with GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA, USA). An analysis of tumor growth was carried out using the two-way analysis of variance (ANOVA) test. One-way ANOVA with Bonferroni's multiple comparison test was used for analysis of the other experiments. A P value of <0.05 was considered to be statistically significant. The combinational antitumor effect was evaluated by the fractional product method.[27,28] The fractional tumor volume (FTV) was calculated as the ratio between the mean tumor volume of each treated group divided by the mean tumor volume of the control group. The expected FTV was calculated by FTV_{L-1MT} multiplied by FTV_{APO866}. The FTV ratio was obtained by dividing the expected FTV by $FTV_{L-1MT+APO866}$. A ratio >1 indicates that there was a synergistic effect, a ratio <1 indicates that there was an antagonistic effect and a value of 1 indicates that the combinational treatment was additive.

Results

L-1MT enhanced the therapeutic effect in the gastric and bladder tumor model via a T-cell response

We hypothesized that blockage of the NAD biosynthesis pathway and activation of immune responses could have a synergistic antitumor effect. We examined the endogenous expression of NAMPT in mouse gastric cancer (MGCC3I), bladder tumor (MBT-2), liver tumor (ML-1) and colon tumor (CT-26) cell lines. The expression of NAMPT was detected in all four of the cell lines, and a higher expression was specifically observed in the MGCC3I and MBT-2 cell lines. Therefore, these two cell lines were chosen for this study (Figure 1a). MGCC3I or MBT-2 cells at a 1×10^6 concentration were inoculated subcutaneously into ICR mice or C3H/HeN mice, respectively. APO866 at a concentration of 25 mg/kg was injected intraperitoneally three times at days 7, 14 and 21, while 5 mg/mL of L-1MT was supplied in the drinking water from days 7 to 21 (Figure 1b). The mean tumor volume was reduced after treatment of either APO866 or L-1MT in the MGCC3I and MBT-2 tumor models. A combination of APO866 and L-1MT further improved the antitumor effect in both of the tumor models ($P = 0.0253$ in the MGCC3I model and $P < 0.001$ in the MBT-2 model) (Figure 1c and d). The combinatorial antitumor effect was evaluated by the fractional product method. The ratio of FTV indicated that L-1MT and APO866 treatments had a near-additive effect in the MGCC3I tumor model and a synergistic effect in the MBT-2 tumor model.

APO866 was reported to have an antiangiogenesis effect in murine renal carcinoma.[7] The number of $CD31^+$ blood vessels significantly decreased in both of the tumor models after APO866 injection. It is interesting to note that the L-1MT supplement reduced the number of blood vessels in the MBT-2 models, but not in the MGCC3I models (Figure 2a–d).

In previous reports, tryptophan depletion suppressed the proliferation of T-cells *in vitro* and *in vivo*.[13,14] To observe the effects on T-cells, we performed an immunohistochemistry assay on tumor samples. The number of $CD8^+$ and $CD4^+$ T-cells in the MGCC3I and MBT-2 tumors significantly increased after L-1MT treatment. In addition, the number of T-cells was not altered significantly in the L-1MT plus APO866 group (Table 1 and Figure 3a–d). This result suggests that APO866 did not significantly disrupt activation of the immune system by L-1MT in animal models.

Evaluating enzyme activity of IDO and NAMPT

Because downstream metabolites of tryptophan might contribute to NAD biosynthesis in cancer cells,[24] we examined the enzyme activities of IDO and NAMPT, as well as the NAD expression level in cells treated with APO866 and L-1MT. The IDO enzyme activity was monitored by kynurenine production. A low IDO enzymatic activity in MGCC3I was observed, but the activity was induced by interferon-γ. A low production of kynurenine in MBT-2 was detected with or without interferon-γ treatment (Figure 4a). To investigate whether downstream metabolites of tryptophan

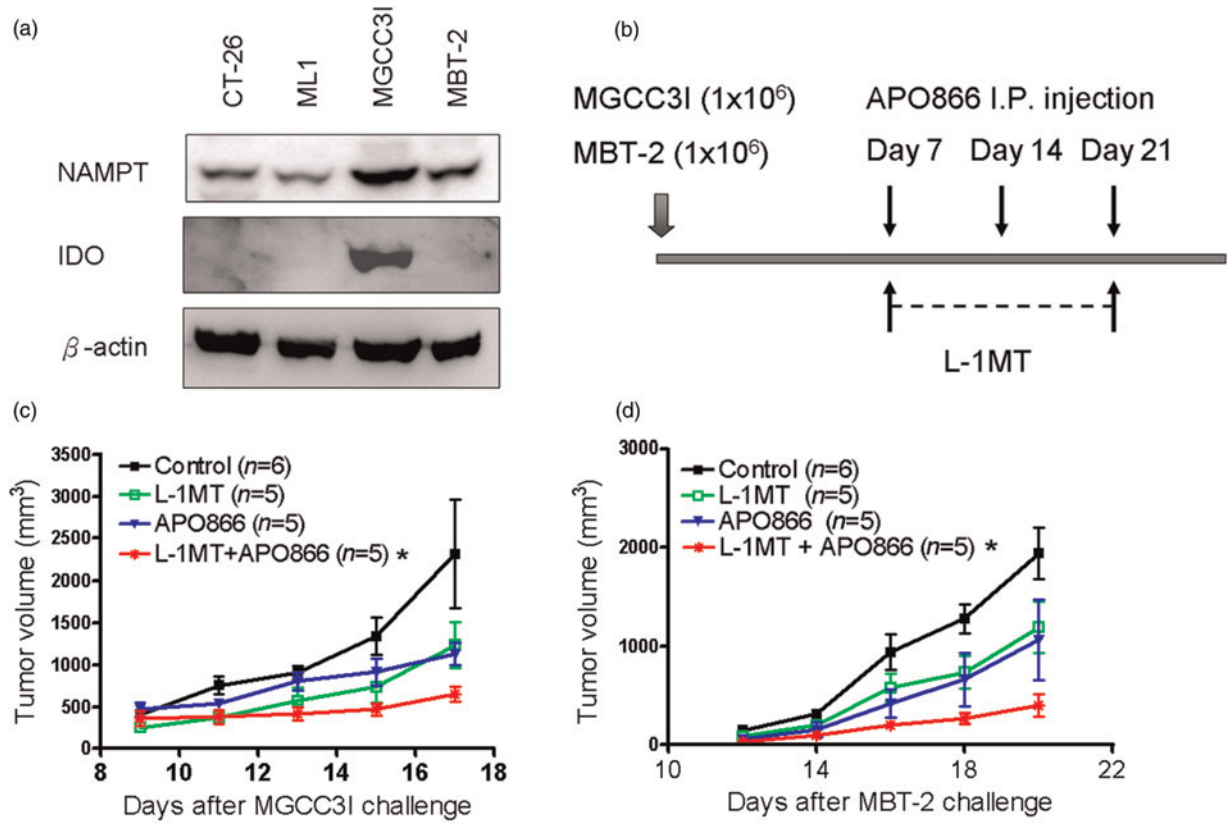

Figure 1 Combining L-1MT and APO866 induced a cooperative antitumor effect in immune-competent mice. (a) NAMPT protein expression in four tumor cell lines. Twenty-five micrograms of cell lysate from normal culture conditions was loaded into each well, and the levels of NAMPT and β-actin were determined by Western blot. Three independent experiments were performed. (b) Protocol for administration of L-1MT and APO866. A concentration of 25 mg/kg of APO866 was injected intraperitoneally into mice at weekly intervals. L-1MT at a concentration of 5 mg/mL was supplied with the drinking water for two weeks. The tumor sample was collected at day 21. (c) Growth curve of the MGCC3I tumor in the ICR mice under APO866 and L-1MT treatment. (d) Growth curve of the MBT-2 tumor in the C3H/HeN mice. The digit in the parenthesis is the number of mice in the experiment. $^*P < 0.05$ versus control (two-way ANOVA). L-1MT, L-1-methyl tryptophan; NAMPT, nicotinamide phosphoribosyl transferase; ANOVA, analysis of variance. (A color version of this figure is available in the online journal)

contribute to NAD production, 500 μmol/L of L-1MT or 5 nmol/L of APO866 was added to the culture media. NAD production was significantly inhibited by APO866 in the MBT-2 and MGCC3I cells ($P = 0.0154$ and 0.0011, respectively). In contrast, NAD production was not inhibited by L-1MT, even in MGCC3I cells treated with interferon-γ ($P = 0.705$). A combination of L-1MT and APO866 did not provide any additional inhibition of NAD production in both of the cell lines (Figure 4b and c). In addition, blocking the tryptophan metabolic pathway did not decrease the intracellular NAD production by these tumor cells *in vitro*. The results suggest that the additive therapeutic effect in the combination therapy was not due to an increase in NAD depletion in the cells.

L-1MT enhanced the therapeutic effect primarily through immune response regulation

Because the immune response is suppressed by IDO-expressing dendritic cells,[14] systematic L-1MT treatment might lead to T-cell proliferation or activation. To investigate whether L-1MT could enhance the therapeutic effect of APO866 in the absence of T-cells, tumor growth was measured in the immune-deficient (NOD/SCID) mice. The mean tumor volume of the APO866 group was smaller than the control and LL-1MT groups. The combination group did not have a better therapeutic effect than the APO866 group alone (Figure 5a and b). This result suggests that the L-1MT-induced therapeutic effect on both of the tumor models was dependent on the presence of a competent immune system.

To rule out the possibility that a high dose of L-1MT induced a cytotoxic effect on the cancer cells, a cell viability assay was performed. Cell growth was not affected after a 500 μmol/L L-1MT treatment, but was significantly attenuated in the MGCC3I and MBT-2 cells after a 5 nmol/L APO866 treatment. The combination treatment group did not have a significant decrease of viability when compared with the APO866 group (Figure 6a and b). Taken together, these data suggest that the additive therapeutic effect is due to the growth-inhibitory effect of APO866 and the immune-activation effect of L-1MT.

Discussion

The manipulation of cellular energy uptake has been one of the strategies for cancer therapy. APO866 is a potent antitumor drug that induces cell death by reducing the

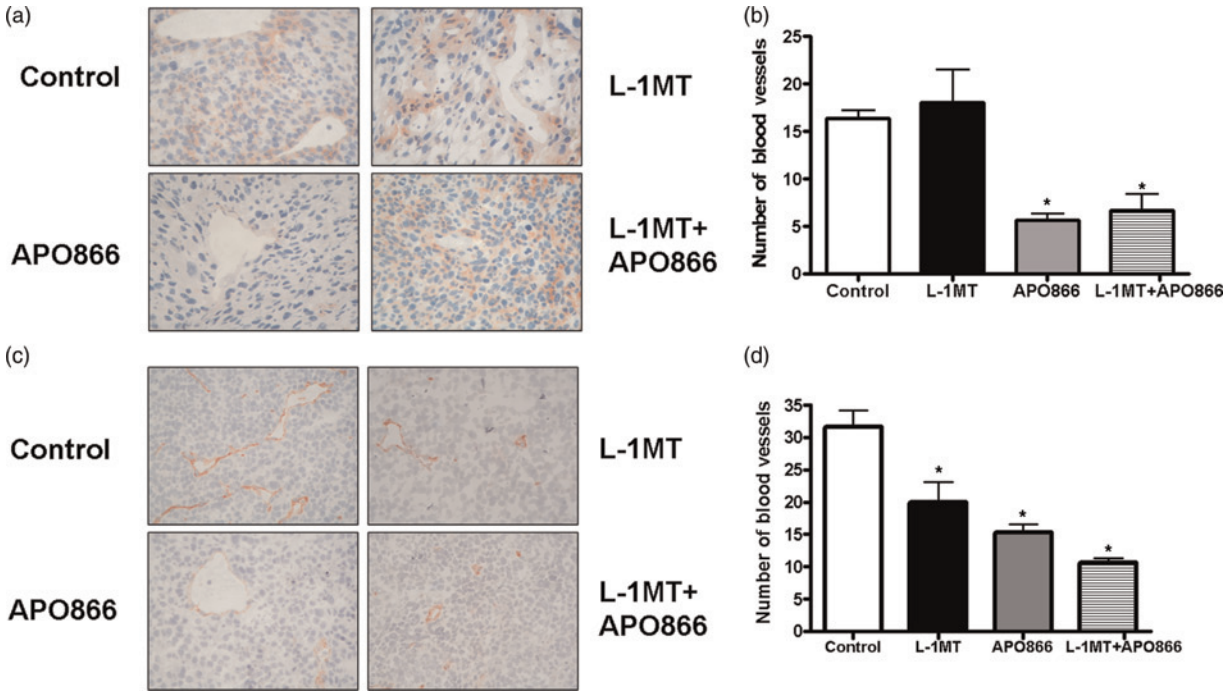

Figure 2 APO866-induced anti-angiogenesis effect. (a) $CD31^+$ blood vessels of the MGCC3I tumor sections were analyzed by immunohistochemistry at a 400× magnification. (b) Quantification of $CD31^+$ blood vessels within the MGCC3I tumor. Five random fields were counted from three independent samples at a 100× magnification (one-way ANOVA, $P = 0.0046$). (c) $CD31^+$ blood vessels of the MBT-2 tumor sections. (d) The number of $CD31^+$ blood vessels in the MBT-2 tumor (one-way ANOVA, $P = 0.0006$). $^*P < 0.05$ versus control, Bonferroni's multiple comparison test. L-1MT, L-1-methyl tryptophan; ANOVA, analysis of variance. (A color version of this figure is available in the online journal)

intracellular level of NAD.[9,10] On the other hand, L-1MT delays tumor growth in several tumor models by reactivating the immune system.[13,18] In the present study, we have demonstrated that a combination of L-1MT and APO866 improved the therapeutic effect in two animal tumor models. Additionally, L-1MT did not significantly alter the intracellular level of NAD and the cell viability in MGCC3I and MBT-2 cells *in vitro*. Our results support the notion that a combination of NAD depletion and immune activation can further enhance the efficacy of cancer therapies *in vivo*.

Previously, it was shown that inhibition of NAMPT by APO866 suppressed the secretion of proinflammatory cytokines in the rheumatoid arthritis animal model *in vivo*.[23] Furthermore, the development of T and B lymphocytes was affected in NAMPT conditional knockout mice.[22] APO866 might prevent T lymphocyte proliferation *in vitro* and attenuate syndrome in the experimental autoimmune encephalomyelitis model.[29] NAMPT is also involved in differentiation of myeloid cells.[30] This evidence implied that immune activation might be affected by NAMPT inhibition. Importantly, our results indicated that the L-1MT treatment enhanced the antitumor effect of APO866 in immune-competent mice, while APO866 did not attenuate immune activity at the doses tested in our experiments. The function of T-cells in the tumor-bearing host was apparently not suppressed by systematic APO866 treatment. The infiltration of T-cells was not significantly different between the APO866 plus L-1MT group and the L-1MT-alone group. Therefore, under appropriate APO866 treatment, the T-cell response was unaffected. In previous reports, tumor-bearing mice received APO866 by intraperitoneal injection two times a day.[8,31] In our research model, APO866 was only injected at weekly intervals. The difference in dosage might explain the limited interference of the immune system in this report, exhibited by infiltrated immune cells at the tumor site.

In a previous study, APO866 was demonstrated to inhibit angiogenesis.[7] Our data support this result because fewer blood vessels were observed in the MBT-2 and MGCC3I tumors after APO866 treatment. Interestingly, L-1MT could inhibit $CD31^+$ blood vessel formation in the MBT2 animal tumor model, but not in the MGCC3I gastric cancer animal model. Although tryptophan depletion might be a factor in the regulation of angiogenesis,[32] L-1MT did not suppress angiogenesis in previous studies. The discrepancy in the MBT-2 and MGCC3I tumor models might be due to the interaction between immune

Table 1 Tumor-infiltrated immune cells

	MGCC3I tumor		MBT-2 tumor	
	CD4	CD8	CD4	CD8
Control	3 ± 1	1 ± 1	7 ± 3	1 ± 1
L-1MT	$16 \pm 4^*$	$12 \pm 4^*$	$42 \pm 9^*$	$7 \pm 3^*$
APO866	1 ± 1	0	$32 \pm 5^*$	1 ± 1
L-1MT + APO866	$13 \pm 5^*$	$10 \pm 4^*$	$27 \pm 11^*$	$6 \pm 3^*$

L-1MT, L-1-methyl tryptophan

Note: The total number of infiltrated T-cells for random five fields was counted under a 400× light microscope, and mean ± standard deviation showed the average of three independent experiments. *Statistically significant difference compared with control group ($P < 0.05$)

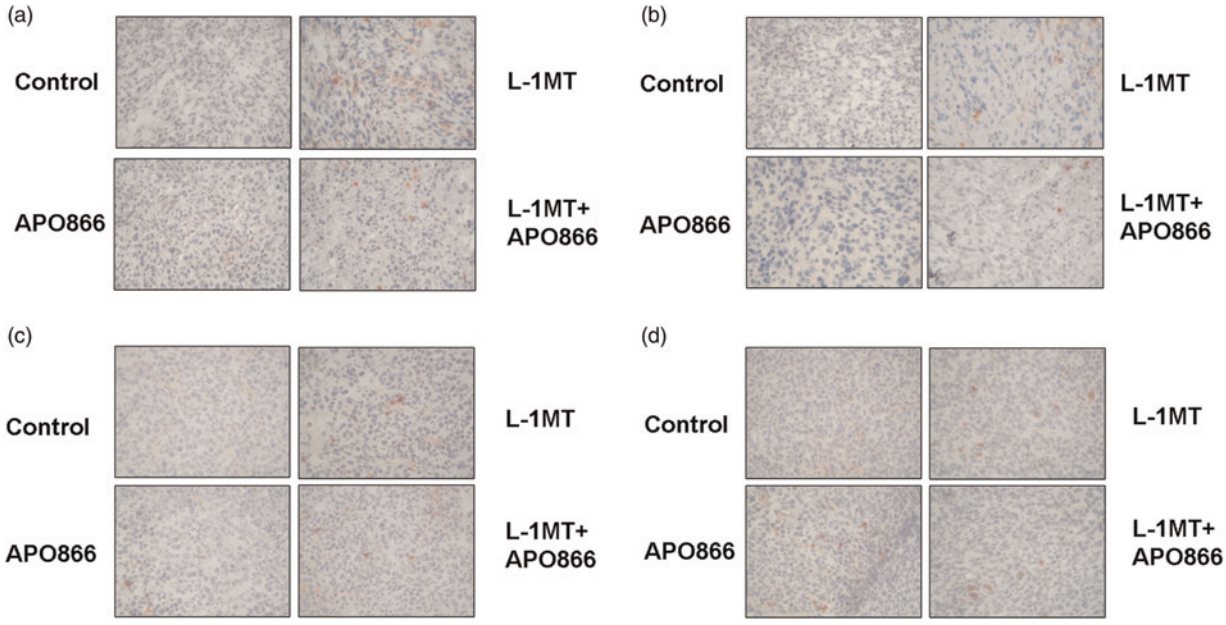

Figure 3 Tumor infiltration of $CD4^+$ and $CD8^+$ T-cells. Analysis of (a) $CD8^+$ and (b) $CD4^+$ T-cells in the MGCC3I tumor section. (c) $CD8^+$ and (d) $CD4^+$ T-cells in the MBT-2 tumor section. The tumor samples were collected at day 20 after tumor inoculation. Each group contained three independent samples. (A color version of this figure is available in the online journal)

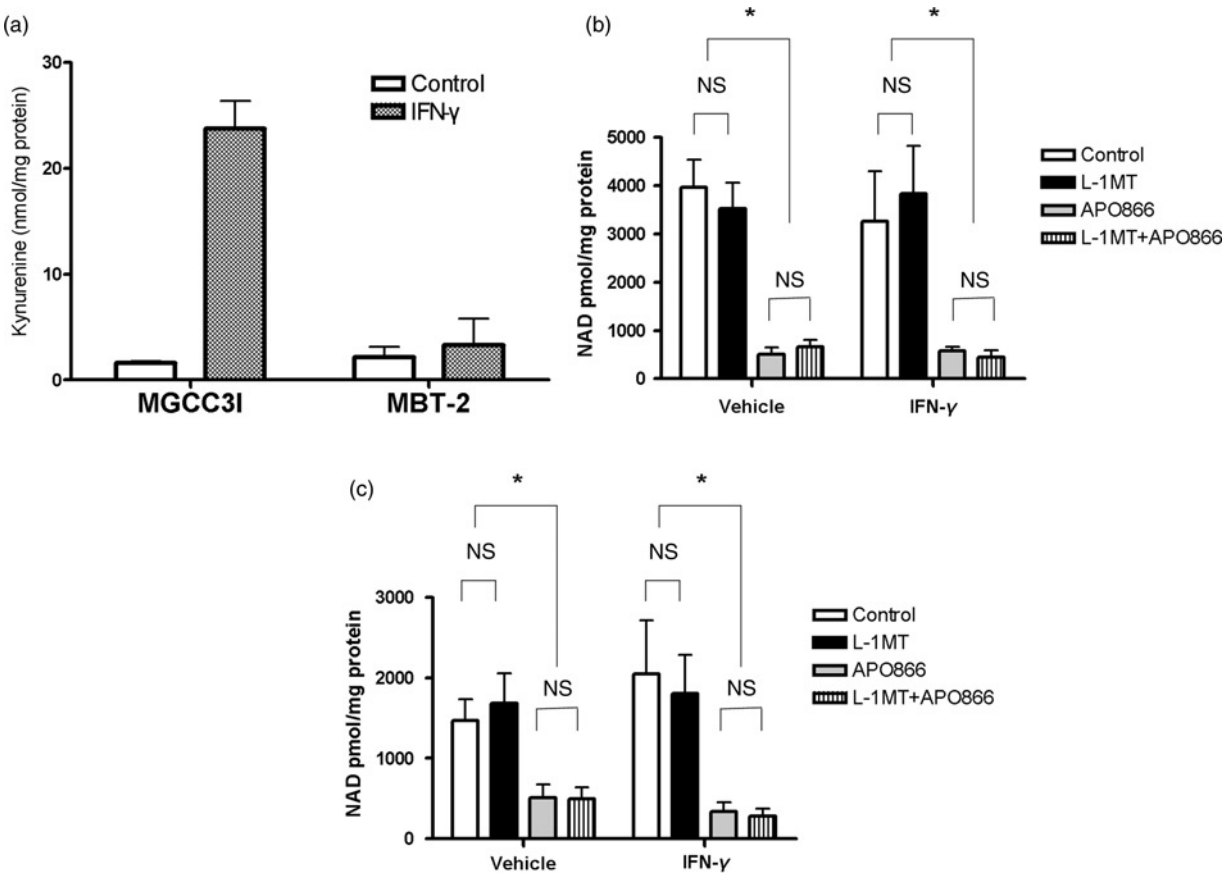

Figure 4 IDO and NAMPT activities during treatment with various inhibitors in the MGCC3I and MBT-2 cells. (a) Enzyme activity of IDO. The total protein was extracted after treatment with the vehicle control or 200 U of interferon-γ for 24 h, and then assayed for the conversion of tryptophan into kynurenine. (b) and (c) enzyme activity of NAMPT. The intracellular NAD concentration was determined in the vehicle media or 200 U of interferon-γ media 24 h after the addition of the vehicle control, 500 μmol/L L-1MT, 5 nmol/L APO866 or the combined treatment in (b) the MGCC3I cells and (c) MBT-2 cells. The symbol NS indicates no significant difference between the two groups. *Statistically significant difference compared with the control group (one-way ANOVA, $P = 0.0006$) ($^*P < 0.05$, Bonferroni multiple comparison test). Three independent experiments were performed. L-1MT, L-1-methyl tryptophan; NAD, nicotinamide adenine dinucleotide; IDO, indoleamine 2,3-dioxygenase; NAMPT, nicotinamide phosphoribosyl transferase; ANOVA, analysis of variance

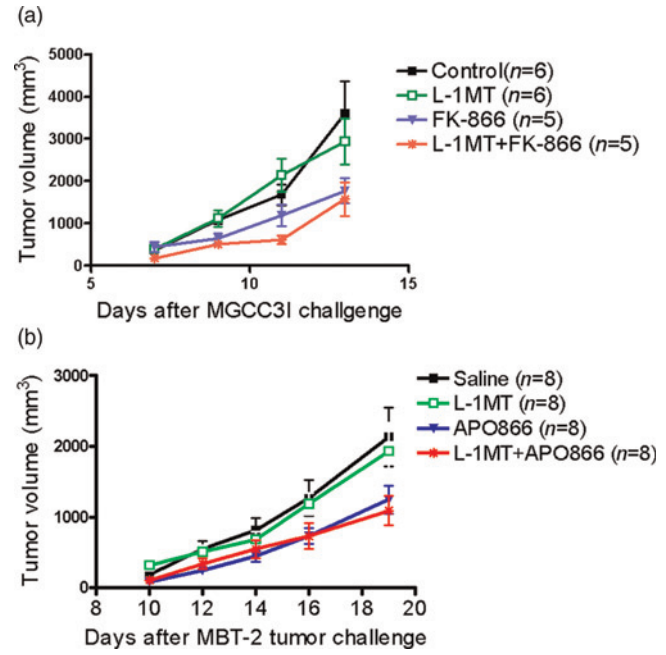

Figure 5 L-1MT did not inhibit tumor growth in immune-defective mice *in vivo*. (a) MGCC3I tumor growth curve in NOD/SCID mice. (b) MBT-2 tumor growth curve in NOD/SCID mice. The digit in the parenthesis is the number of mice in the experiment. L-1MT, L-1-methyl tryptophan; NOD/SCID, non-obese diabetes/severe combined immunodeficiency. (A color version of this figure is available in the online journal)

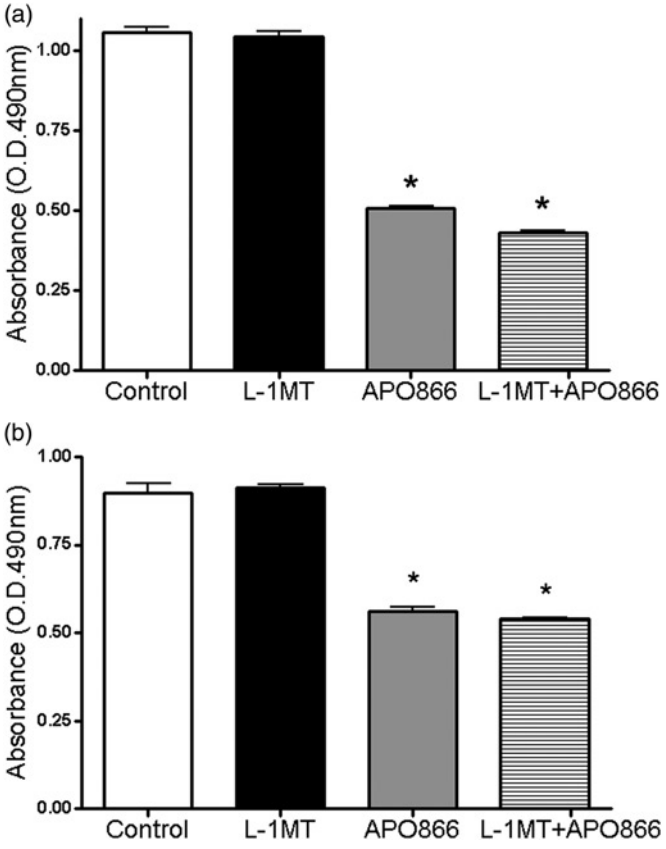

Figure 6 L-1MT did not inhibit cell growth *in vitro*. The effects of L-1MT, APO866 or the combination treatment on the cell viability of (a) MGCC3I cells and (b) MBT-2 cells. Three independent experiments were performed. *Statistically significant difference when compared with the control group ($P < 0.05$). L-1MT, L-1-methyl tryptophan

cells and cancer cells. Fewer T-cells infiltrated the MGCC3I tumor than the MBT-2 tumor under the same treatment, shown in Table 1. The tumor-infiltrating T-cells and cancer cells might release cytokines or chemokines to recruit other immune cells into the tumor. Various studies have indicated that the tumor-associated macrophages or neutrophils are involved in the process of angiogenesis.[33,34] Thus, T-cells, macrophages and neutrophils might be the factors responsible for the antiangiogenesis that is induced by L-1MT in the MBT-2 animal tumor model. However, we cannot exclude the possibility of insufficient uptake of L-1MT *in vivo*. The IDO enzyme was significantly elevated by interferon-γ in the MGCC3I cells, and the concentration of L-1MT in the serum and within the tumor might not completely block IDO activity *in vivo*. The detailed mechanism of antiangiogenesis induced by L-1MT requires further investigation.

The phase I clinical trial of CHS 828, another NAD-specific inhibitor, revealed that there was a limited therapeutic efficacy on patients.[35] In addition, APO866 did not show objective responses in a clinical report.[36] L-1MT or APO866 alone did not show any significant antitumor effects in the animal tumor models. L-1MT enhanced the therapeutic effects of APO866 *in vivo*, primarily through modulation of immune responses. The concentration of APO866 used in this study selectively suppressed tumor growth without profoundly interfering with the immune system. Therefore, our results provide a new strategy for the clinical development of combinatorial cancer therapy with IDO and NAMPT inhibitors.

Author contributions: The contributions of each author to this study were as follows: H-JY, M-CY, C-CL, Y-LC, T-YW and T-TH contributed to collecting data, data analysis and interpretation. C-ML helped data analysis. The concept and experimental design was from H-JY and M-DL. The manuscript was drafted by M-CY and M-DL. H-JY and M-CY contributed equally to this work. We thank Dr C-LW and Dr Shih-Yao Chen for their assistance with statistical analysis. All authors read and approved the final manuscript.

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