1-L-MT, an IDO inhibitor, prevented colitis-associated cancer by inducing CDC20 inhibition-mediated mitotic death of colon cancer cells

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Novelty and Impact statements

We firstly demonstrated the chemopreventive effects of 1-L-MT on colitis-associated cancer, which were independent of adaptive immunity. IDO inhibition in colon cancer cells suppressed CDC20 transcription, thus inducing mitotic death, which was mediated by decreased Kyn production. We identified IDO/ Kyn pathway as a preventive target for colorectal cancer and other malignancies.

Abbreviation:

IDO: Indoleamine 2,3 dioxygenase; 1-L-MT: 1-L-methyltryptophan; Tregs: Regulatory T cells; Kyn: kynurenine; CRC: Colorectal carcinoma

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Abstract
Indoleamine 2,3-dioxygenase 1 (IDO1), known as IDO, catabolizes tryptophan through kynurenine pathway, whose activity is correlated with impaired clinical outcome of colorectal cancer. Here we showed that 1-L-MT, a canonical IDO inhibitor, suppressed proliferation of human colorectal cancer cells through inducing mitotic death. Our results showed that inhibition of IDO decreased the transcription of CDC20, which resulted in G2/M cycle arrest of HCT-116 and HT-29. Furthermore, 1-L-MT induced mitochondria injuries and caused apoptotic cancer cells. Importantly, 1-L-MT protected mice from azoxymethane (AOM)/dextran sodium sulfate (DSS)-induced colon carcinogenesis, with reduced mortality, tumor number and size. What is more, IDO1/-/ mice exhibited fewer tumor burdens and reduced proliferation in the neoplastic epithelium, while, 1-L-MT didn't exhibit any further protective effects on IDO/-/ mice, confirming the critical role of IDO and the protective effect of 1-L-MT-mediated IDO inhibition in CRC. Furthermore, 1-L-MT also alleviated CRC in Rag1/-/ mice, demonstrating the modulatory effects of IDO independent of its role in modulating adaptive immunity. Taken together, our findings validated that the anti-proliferation effect of 1-L-MT in vitro and the prevention of CRC in vivo were through IDO-induced cell cycle disaster of colon cancer cells. Our results identified 1-L-MT as a promising candidate for the chemoprevention of CRC.

Introduction
Colorectal cancer (CRC), one of the most common cancers, remains the third leading cause of cancer-related death in the United States \(^1\). Its etiology was demonstrated to be linked to genetic mutations, cancer-associated inflammation, epigenetic abnormalities and so on \(^2-4\). Recent study reported that in the stage I of CRC, 50% cases were positive for indoleamine 2,3- dioxygenase (IDO) staining, which could be a predictor of distant metastasis \(^5\). Higher IDO expression and activation are involved in CRC progression, and correlated with poor clinical outcome \(^6\). What is more, it was reported that systemic administration of Salmonella inhibited tumor growth and extended the survival of mice bearing colon cancer via suppressing IDO activation \(^7\).
Although IDO expression has been reported in human CRC, the function of IDO activity in this disease remains unclear.

IDO participates in pathogenic inflammation and engenders immune tolerance to tumor antigens. IDO degrades tryptophan and produces many toxic kynurenine (Kyn) metabolites, which mediate immune tolerance. The function of IDO involves multiple effects on T lymphocytes, including inhibiting activated T cells proliferation, inducing apoptosis of CD8+ T cells, and promoting differentiation of CD4+ T cells towards a regulatory phenotype. Nowadays, more attention has been focused on the effects of IDO independent of its role in adaptive immunity. It was demonstrated that blockade of IDO activity in cancer cells could reduce β-catenin activation and inhibit cell proliferation. Knockdown of IDO decreased the formation of vasculogenic mimicry of lung cancer. Silence of IDO in melanoma cells inhibited cancer cell proliferation and induced greater cell apoptosis. IDO could directly affect tumor cells, including blocking mTOR activity, inducing autophagy and increasing NAD+ levels. In our study, we found that IDO inhibitors significantly induced mitotic death in HCT-116 and HT-29 colon cancer cells. Thus, apart from immune tolerance, IDO activation contributed pathologically to tumorigenesis of colon cancer.

IDO has been regarded as an attractive target for both prevention and treatment of many cancer. 1-MT is a canonical IDO inhibitor with low toxicity, which is well tolerated by cancer patients. Moreover, 1-MT, combined with several chemotherapeutic drugs, resulted in rapid eradication of established malignancies. What is more, Kengo Ogawa et al. identified that 1-L-MT, an isomer of 1-MT, suppressed AOM-induced colonic preneoplastic lesions in rats. 1-L-MT, but not 1-D-MT exhibited greater effects on anti-proliferation of colon cancer cells. Consistently, AOM/DSS-induced sporadic colon cancer was prevented by 1-L-MT administration in our in vivo study. Hence, we assumed that 1-L-MT could potentially be used for chemoprevention against colitis-associated cancer.
In the present study, we validated that 1-L-MT inhibited proliferation of human colorectal cancer cells through inducing cell cycle arrest-mediated apoptosis. Importantly, supplementing the diet with 1-L-MT protected mice against AOM/DSS-induced colon carcinogenesis independent of its role in adaptive immunity.

**Result**

**IDO inhibitors suppressed the proliferation of colon cancer cells**

To investigate the possible role of IDO in colon cells, two IDO inhibitors, 1-L-MT and Epacadostat (INCB024360) were used. As shown in Fig. 1A, B and Supplementary Fig. 1A, B, different doses of IDO inhibitors significantly suppressed the viability of two colon cancer cell lines, including HCT-116 and HT-29 cells. INCB exhibited better effects than 1-L-MT. Stimulation of 1-L-MT (5mM) or INCB (50µM) for both 24h and 48h remarkably downregulated IDO activity with reduced Kyn synthesis (Supplementary Fig. 1C) and kynurenine acid secretion (Fig. 1C). Plate clone formation assay further confirmed that IDO inhibition in colon cancer cells indeed suppressed their proliferation (Supplementary Fig. 1D). Altogether, these data demonstrated that both 1-L-MT and INCB inhibited colon cancer cell proliferation in vitro.

**Long time exposure to IDO inhibitors led to apoptosis of colon cancer cells**

As the cell viability was inhibited by 1-L-MT and INCB, we next detected whether apoptosis or cell cycle was affected by IDO inhibition. There was no apoptotic cells observed with stimulation of 1-L-MT and INCB at 24h, while long time exposure (48h) to either 1-L-MT or INCB remarkably induced apoptotic cell death, including early and late apoptosis (Fig. 1D). Cell cycle distribution of colon cancer cells was measured and a G2/M phase arrest occurred when cells were treated with IDO inhibitors for both 24 and 48h (Fig. 1E, F).
IDO inhibition induced mitotic cell accumulation in colon cancer cells

As shown in Fig. 2A, the percentage EdU-positive HCT116 and HT-29 cells were reduced by IDO inhibitors treatment, verifying the proliferative suppression induced by 1-L-MT and INCB. Spindle checkpoint machinery (SAC) controls the fidelity of chromosome segregation in mitosis and maintains proper mitotic exit and Mad2, CDC20 and BubR1 are vital components of the SAC. As shown in Fig. 2B-E, IDO inhibitors markedly increased the BubR1 and MAD2 expressions, which peaked at 2h to 8h, and then gradually decreased. While, the protein level of CDC20 was suppressed dramatically at 24h. In order to determine whether IDO inhibitors-treated cells were arrested at the mitotic phase, HCT-116 and HT-29 cells were stained with p-histone H3 (a mitotic marker) and PI. As seen in Fig. 2F, mitotic cells were increased by IDO inhibitors. Fig. 2G-J showed that IDO inhibition-mediated mitotic cell accumulation wasn't affected by silence of either BubR1 or MAD2, indicating that the effects of 1-L-MT and INCB on mitotic cell accumulation did not involve MAD2 or BubR1.

IDO inhibitors induced mitotic death of colon cancer cells via suppressing CDC20 expression

As shown in Fig. 3A, B, the degradation of cyclin B1, and following inhibition of CDK, with phosphorylation at Thr161 and dephosphorylation at Tyr15, were suppressed by IDO inhibitors. 1-L-MT and INCB promoted the maintenance of microtubule polymerization with increased microtubule density (Fig. 3C), which was similar to paclitaxel (a microtubule polymerizing agent). As shown in Figure 3A, B, treatment with IDO inhibitors induced a marked decrease soluble in α-tubulin and a commensurate marked increase in insoluble α-tubulin, indicating that microtubule polymerization was maintained in colon cancer cells when exposed to IDO inhibitors. To further determine the relationship between mitotic arrest and cell death, immunoblotting was performed for PARP, which is cleaved during apoptosis, and for MPM2, which recognizes mitotic phosphoproteins (Fig. 3D-G). PARP cleavage in HCT-116 and HT-29 was coincident with strong MPM2 staining, suggesting that IDO
inhibition contributed to mitotic death. BubR1 and MAD2 (Fig. 3H, I) were not involved in the IDO inhibitor-induced mitotic cell death. While CDC20 was required in the regulation of IDO on mitotic death of colon cancer cells (Fig. 3J-M). Taken together, these results revealed that CDC20 played a critical role in IDO inhibitors-induced mitotic death of colon cancer cells.

IDO inhibition resulted in mitochondrial damage and caspase-dependent apoptosis
A remarkable ROS accumulation was induced by both 1-L-MT and INCB, detected by DCFH-DA staining (in Supplementary Fig. 2A, B). JC-1 dye was adopted to test mitochondrial membrane potential (MMP). Significant enhancement of green fluorescence, indicating a decrease in MMP, was caused by IDO inhibition (Supplementary Fig. 2C). Moreover, Cytochrome C released was increased by treatment of IDO inhibitors in a time dependent manner, confirming that mitochondrial injuries were caused by IDO suppression (Supplementary Fig. 2D-F). Accordingly, 1-L-MT and INCB led to the activations of caspases in colon cancer cells, with increased levels of cleaved caspase-3, -8 and-9, and decreased level of BCL-2 expression (Supplementary Fig. 2G-J). To validate a role of CDC20 in IDO inhibitors-mediated apoptosis, CDC20 siRNA was used to intervene CDC20 expression. No remarkable changes of cleaved caspase-3, -8, -9 and BCL-2 were observed in CDC20 siRNA-transfected cells with or without IDO inhibitors treatment (Supplementary Fig. 2K, L). In conclusion, IDO inhibitors induced apoptosis of colon cancer cells via suppressing CDC20.

Kyn rescued IDO inhibition-induced mitotic cell death
Reduced Ki67 level induced by 1-L-MT and INCB was abolished by Kyn, a major metabolite of IDO, as shown in Supplementary Fig. 3A. MTT assay reflected that Kyn stimulation reversed the decreased viability of both HCT-116 and HT-29 cells caused by IDO inhibitors (Supplementary Fig. 3B). Similarly, cell cycle arrest-mediated apoptosis (Supplementary Fig. 3C, D) and the change of associated proteins (Supplementary Fig. 3E, F) by 1-L-MT and INCB was compromised by Kyn
stimulation. To pinpoint whether IDO regulated colon cancer cell proliferation via Kyn, IDO siRNA was used to suppress endogenous IDO expression (Supplementary Fig. 3G), and Kyn stimulation rescued the decreased expression of Ki67 in colon cancer cells (Supplementary Fig. 3H, I). These results suggested that IDO-mediated Kyn was required for colon cancer cells proliferation.

AhR was dispensable in Kyn-induced CDC20 transcription

We assessed the effects of Trp, Kyn and INF-γ on HCT-116 and HT-29 cell viability using MTT assay. As shown in Fig. 4A, Trp had no impact on the viability of HCT-116 and HT-29 cells. And Trp stimulation hardly affected expressions of mitotic death-related proteins. While Kyn enhanced CDC20-mediated cyclin B1 degradation and CDK inhibition (Fig. 4B, C), which accelerated the cell cycle and proliferation. Furthermore, both activation of IDO by INF-γ and increased level of its metabolite, Kyn, remarkably promoted CDC20 transcription (Fig. 4D). It is known that aryl hydrocarbon receptor (AhR), a ligand-operated transcription factor, was a major receptor of Kyn. We then explored the potential role of AhR, and found that its protein level could neither be changed by IDO inducer nor inhibitors (Fig. 4E). Consistently, 1-L-MT and INCB also inhibited CDC20 expression in AhR-silenced HCT-116 and HT-29 cells, confirming that AhR wasn't required in the regulation of IDO on CDC20 (Fig. 4F, G). Moreover, the nuclear translocation of AhR wasn't affected by IDO inhibitors (Fig. 4H). And when AhR activation was inhibited by CH223191, 1-L-MT and INCB still suppressed CDC20 expression, which further validated that AhR was dispensable in this process (Fig. 4I). Interestingly, we found that Kyn could translocate into nucleus of colon cancer cells (Fig. 4J). There may be other nuclear transcription factors mediating CDC20 transcription, which should be explored in further. Taken together, Kyn promoted transcription of CDC20 independent of AhR activation.

1-L-MT prevented colitis-associated tumorigenesis

To further test whether the in vitro biological effects of IDO inhibition on colon
cancer cells were also true in vivo, an AOM/DSS-induced CAC model was used (Fig. 5A). Relative body weight of the mice was recorded during the experiment (Fig. 5B). 1-L-MT decreased weight loss of mice comparing with AOM/DSS group. Based on Kaplan-Meier survival curves, administration of 1-L-MT increased the survival of mice (Fig. 5C). Average tumor size in 1-L-MT groups was smaller than that of AOM/DSS group (Fig. 5D, E). Consistently, non-polypoid dysplasia was also attenuated with administration of different doses of 1-L-MT (Fig. 5F). What is more, histology score was reduced by 1-L-MT administration, represented by clinical parameters containing weight loss, stool consistency and bleeding, respectively (Fig. 5G). Compared with the AOM/DSS group, there was a remarkable increase in colon length of mice in the 1-L-MT groups (Fig. 5H). Hence, the data strongly revealed that a diet supplemented with 1-L-MT prevented colorectal tumorigenesis in a mouse model of CRC.

1-L-MT suppressed IDO/Kyn pathway in CRC

We next explored the activity of IDO in this CRC model. INF-γ (Supplementary Fig. 4A, I), Trp (Supplementary Fig. 4B, J) and Kyn (Supplementary Fig. 4C, G) in serum and colon section were measured. It was found that 1-L-MT remarkably inhibited AOM/DSS-induced elevated levels of INF-γ and Kyn. Meanwhile, the enhanced ratio of Kyn to Trp was blocked by different doses of 1-L-MT (Supplementary Fig. 4D, H). As shown in Supplementary Fig. 4E, the mRNA levels of IDO and CDC20 were notably suppressed by 1-L-MT in tumor, but not in the mucosa of the colon section, which is consistent with their protein expressions (Supplementary Fig. 4F). However, INF-γ mRNA expressions were similar among all groups. Immunochemistry assay reflected that AOM/DSS-induced expressions of IDO, Kyn and CDC20 were significantly suppressed by 1-L-MT (Supplementary Fig. 4K). Apoptosis in tumor tissues in 1-L-MT groups was evaluated shown by TUNEL assay, indicating that apoptosis index of tumor with lower IDO expression was much higher than that of AOM/DSS group (Supplementary Fig. 4M). However, comparing with AOM/DSS group, a little decreased expression of Ki67 and increased expression of caspase-3.
were observed in 1-L-MT treatment groups. These results suggested that 1-L-MT inhibited IDO activation and induced apoptotic cells in the tumor of colon section.

The effects of 1-L-MT on CRC were dependent on IDO
To further test whether the effects of 1-L-MT were mediated by IDO inhibition, AOM/DSS-induced CRC model were built in IDO/-/- mice. Comparing with WT, IDO/-/- mice harbored fewer and smaller tumors in colon. 1-L-MT (200mg/kg) didn't show any further protective effects in IDO/-/- mice (Fig. 6A, B). There was almost no differences in tumor number and size between IDO/-/- group and 1-L-MT group. The mortality in WT group was higher than that in IDO/-/- group (Fig. 6C). Histological analysis exhibited that erosion and distortion of crypts and a non-polypoid dysplasia were also similar in IDO/-/- mice with or without 1-L-MT administration (Fig. 6D, E). AOM/DSS induced higher Kyn to Trp ratio, and IDO deficiency abolished this enhancement, while 1-L-MT didn't cause any further suppression (Fig. 6F). In the meantime, we found that the increased protein levels of IDO, CDC20 and Kyn in WT group could hardly be detected by Western blot and Immunohistochemistry in IDO/-/- mice (Fig. 6G, H). Comparing with WT group, apoptosis could be detected in tumor of IDO/-/- mice, and there wasn't significant difference observed in IDO/-/- mice with or without 1-L-MT treatment (Fig. 6I). Taken together, these results suggested that 1-L-MT alleviated CRC via inhibiting IDO.

1-L-MT prevented CRC and induced tumor apoptosis independent of its effect on adaptive immunity
To identify the non-immunomodulatory effects of IDO inhibition by 1-L-MT, we established CRC model in Rag1/-/- mice, which were absent of T cells. As expected, administration of 1-L-MT (200mg/kg) reduced tumor number and size, which was induced by AOM/DSS (Supplementary Fig. 5A, B). 1-L-MT also rescued the decreased survival rate of Rag1/-/- mice induced by AOM/DSS (Supplementary Fig. 5C). HE staining and histology score further confirmed the effects of 1-L-MT on CRC (Supplementary Fig. 5D, E). As shown in Supplementary Fig. 5F and G, IDO...
activation and CDC20 expression were obviously decreased by 1-L-MT. More apoptotic tumor cells in the colon section were detected in 1-L-MT group than in AOM/DSS group (Supplementary Fig. 5H). These results indicated the chemopreventive effect of 1-L-MT in colitis-associated colorectal cancer did not involve its regulatory effect on adaptive immunity.

**Discussion**

IDO regulates the rate-limiting step of the Kyn pathway of Trp metabolism. Research has been focused on IDO-mediated immune tolerance. It was found that IDO expressing tumor cells regulated the immune escape of themselves. We found that there were more Tregs and fewer CD8+ T cells infiltrated in the colon section of AOM/DSS group, comparing with control group (data not shown). IDO-/− mice harbored fewer tumors, with significantly increased CD8+ T cells and decreased Tregs infiltration. Indeed, IDO mediated immune tolerance contributed to tumor progression in CRC. Interestingly, 1-L-MT also prevented CRC in Rag1-/− mice, which lack T cells. That is, IDO inhibition could suppress cancer development independent of its regulatory effects on adaptive immunity. Besides immune regulation, IDO activation affected tumor cell viability, proliferation and apoptosis in vitro. Consistently, previous study reported that Kynurenine and quinolinic acid (QA), but not picolinic acid, rescued cell proliferation in the IDO blockade in colon cancer cell lines, including HCT 116 cells. We speculated that IDO activation in tumors may have two complementary functions: to mediate tumoral immune tolerance and to accelerate tumor cell proliferation.

The severity of colitis contributes to the development of CRC. The effects of IDO have been investigated in many kinds of colitis models. In TNBS-induced colitis, which is modulated by T cells, IDO, a natural brake, regulated the transdifferentiation of Th1 and Th17 during inflammation. However, the activity of IDO was reported to be dispensable in DSS-induced colitis model. IDO-/− mice showed similar spontaneous intestinal inflammation as that in WT group. While, Matteoli et al.
pinpointed that IDO inhibitor worsened the severity of colitis in the recovery phase of epithelial reconstitution after severe injury with 5% DSS. Together with the current study, these data verified that IDO inhibitors prevented CRC independent of adaptive immunity.

IDO activation-induced localized depletion of Trp regulated T cells function, including apoptosis of CD4+ T cells, and proliferation of Tregs. Moreover, GCN2 could be activated by asparagine or glucose starvation in tumor microenvironment, which was involved in the regulation of immune tolerance. However, our in vivo study reflected that the concentration of tryptophan was similar among Control, AOM/DSS and 1-L-MT administration groups. The stimulation of Trp didn't influence the viability of colon cancer cells. What's more, IDO-induced Trp starvation didn't exhibit any regulatory effects of T cells function, and additional Trp not only could not rescue IDO-mediated T cell inhibition, but also failed to affect colon cancer cell proliferation (data not shown). In summary, tumor-based IDO activation drove progression of CRC independent of essential amino acid starvation.

In the present study, Kyn, a metabolite of Trp, played an essential role in the effects of IDO on colon cancer cells. We found that Kyn increased CDC20 transcription. IDO inhibition blocked this enhancement, and induced cell cycle arrest-mediated apoptosis. Cell cycle checkpoints are critical in the regulation of DNA repair, apoptosis and differentiation. CDC20 is essential in metaphase-to-anaphase transition. Under circumstances of chromosome misalignment, CDC20 can be suppressed by other proteins, including MAD2, resulting in delayed anaphase. While, after correcting chromosomal alignment, CDC20 is activated, and then trigger polyubiquitination and proteasomal degradation of securin and cyclin B. After stimulation of IDO inhibitors, both mRNA and protein levels of CDC20 were decreased. Meanwhile, colon cancer cells, including HCT-116 and HT-29 underwent G2/M cycle arrest, but no obvious apoptosis was detected. After incubation for 48 h, both cell cycle arrest and apoptosis were observed. In order to determine whether IDO induced apoptosis
via cell cycle arrest, cell cycle and apoptosis-related proteins were examined. PARP cleavage, a marker of apoptosis, was coincidently increased with strong MPM2 staining, a key protein in cell cycle, demonstrating that IDO inhibition mediated colon cancer cells death in mitosis. Additionally, 1-L-MT and INCB couldn't induce more apoptosis in CDC20 siRNA-transfected colon cancer cells. These data verified that CDC20, a regulator of cell cycle, mediated cell cycle disaster of colon cancer cells.

Kyn promoted transcription of CDC20 in our study. While AhR, a major receptor of Kyn, wasn't required in CDC20 transcription. When AhR was knockdown or inhibited, 1-L-MT and INCB still inhibited the proliferation of colon cancer cells. Previous study provided evidence that IDO/Kyn/IL-6 axis modulated cell proliferation. PTEN, for example, was a negative regulator of mitotic checkpoint complex during cell cycle via interacting with CDC20 and MAD2. Cytochrome P450 1B1 expression was reported to be associated with CDC20 expression in clinical samples of renal cell carcinoma, which had an impact on IDO/Kyn/AhR pathway. It is still unknown whether there is a crosslink between the IDO pathway and CDC20-associated mitotic arrest. Further in-depth biochemical analysis about the interaction between IDO/Kyn and CDC20-related apoptosis should provide mechanistic insights.

It was reported that kynurenine levels did not always correlate with IDO1 expression, and NAD+ synthesis was not impaired by 1-L-MT In murine tumor models. The expression of Trp 2,3-dioxygenase (TDO) may take part in the progression of CRC. While, in our study, we found that IDO-/ mice showed lower Kyn level (Supplementary Fig. 5G) and administration of Kyn could reverse the effects of IDO deficiency (data not shown). What is more, Kengo Ogawa et al. demonstrated that TDO exerted little effect on L-kynurenine levels, because the expression of TDO was similar between the AOM-treated group and the control group. In our study, we found that 1-L-MT exerted chemopreventive effects mainly dependent on decreased Kyn. We could not exclude the role of TDO, but the specific role of it would need to be explored in further experiments.
It is known that CRC is one of the most commonly diagnosed cancer. Many preventative measures, including dietary and lifestyle modifications, are considered as attractive strategies to reduce the global burden of CRC. Owing to availability and safety, dietary supplements could be a better choice to prevent CRC. IDO upregulation contributed to the early phase of colon carcinogenesis, and 1-MT could potentially be used for chemoprevention of CRC. 1-MT has two racemic isoforms, 1-L-MT and 1-D-MT. 1-L-MT is the classic non-competitive inhibitor of IDO1, and 1-D-MT has been suggested to be less active in inhibiting IDO1, but showing higher potency in target IDO2, whose physiological relevance particularly in humans remains unclear. 1-L-MT was reported to be more effective in abrogating kynurenine generation and tryptophan depletion, thus reversing IDO-induced arrest of human T-cell proliferation. The tissue distributions of 1-L-MT and 1-D-MT are different, with 1-L-MT distributed mainly in pancreas and 1-D-MT in kidney, respectively. Even though 1-D-MT was used to treat several types of cancers in combination with other chemotherapy drugs, the mechanism of it is still unclear. While, there were cell type-specific differences between 1-L-MT and 1-D-MT. Previous study demonstrated that the effect of 1-L-MT was greater than 1-D-MT on anti-proliferation of colon cancer cells. What is more, 1-D-MT was also reported to upregulated IDO in cancer cells. In our study, we found IDO1/- mice harbored fewer tumors in colon section, comparing with WT mice. Thus, we chose 1-L-MT as a chemopreventive agent for colorectal cancer in our present study. What is more, 200 mg/kg and 400 mg/kg 1-L-MT in mice, equivalent to 21.9 and 43.95 mg/kg in humans, are lower than 30 mg/kg body wt twice daily for a 70 kg adult clinically used for 1-D-MT. The exact doses of 1-L-MT to be tolerable and effective in human would require further clinical investigations.

Our results showed that IDO activation played a critical role in CRC progression, which provided mechanistic evidence for the phenomena that high IDO expression was associated with poor prognosis of human CRC. Our investigation provided new
insight into the chemopreventive effects of 1-L-MT, an IDO inhibitor, on CRC. IDO inhibition suppressed tumor growth and induced apoptosis in tumor cells via reducing CDC20 transcription and inducing mitochondrial injuries. These results exhibited important implications for IDO inhibitors as chemopreventive agents for IDO-expressing colitis-associated and sporadic colonic neoplasms. What is more, regulation of the Kyn pathway in the colon section could be regarded as a chemopreventive target for CRC and other malignancies.

Materials and Methods
Reagents
1-L-MT purchased from Sigma-Aldrich was dissolved in 0.1M NaOH at 50mM. INCB purchased from selleck was dissolved in DMSO 5mM. Primary antibodies for IDO, CDC20, β-actin, Lamin A, and horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Bioworld Technology, Inc. AhR antibody were purchased from Abclonal, Boston, Ma, USA. α-tubulin, Mad2 and BubR1 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Cleaved caspase 3, 8, 9, Bcl-2, phospho-histone H3, CDK1 (Tyr15), phospho-CDK1 (Thr161) and CDK1 antibodies were purchased from Cell Signaling Technology (Beverly, MA). Phorbol ELISA kits for Trp, Kyn and INF-γ were purchased from Nanjing SenBeiJia Biological Technology Co., Ltd.

Cell culture
Human colon cancer cell lines (HCT116 and HT29) were obtained from Cell Bank of the Chinese Academic of Sciences (Shanghai, China) and cultured in RPMI 1640 medium supplemented with 10% FBS. Both cell lines were cultured under a humidified 5% (v/v) CO2 atmosphere at 37°C. HCT116 and HT29 were authenticated with methods of short tandem repeat (STR).

Western blot assay
Total proteins were extracted by adding RIPA lysis buffer with 1mM PMSF for 1h on
the ice and centrifuging at 13,000rpm for 30min at 4°C. Protein concentration in the supernatants was measured by BCA protein assay (Thermo). Then, equal amount of sample was run on 10% SDS PAGE. The proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore) using a semidry transfer system (Bio-rad). Proteins were detected using specific antibodies of IDO, BubR1, MAD2, p-Histone, CDC20, Cyclin B1, CDK, p-CDK Thr 161, p-CDK Try 15, tublin, PARP, MPM2 cleaved caspase 3,8,9, Bcl-2 and β-actin overnight at 4°C. The membranes were then washed three times with PBST followed by HRP-conjugated secondary antibodies for 1h at 37°C. All of the antibodies were diluted in PBST containing 1% BSA. The signals were analyzed using the ECL chemiluminescence detection system (Tanon).

Plate colony-formation assay

Cells were seeded in 6-well plates at 100 cells/well in RPMI-1640 culture medium with different stimulations. Plates were further incubated for a week until the colonies were large enough to be visualized. The colonies were pictured at 100 magnification to detect colony size.

Colorimetric MTT Assay

The cytotoxicity was measured by the modified MTT assay. Briefly, the logarithmic cells were plated into 96-well plates at a density of 4000~5000 cells/well in a final volume of 100µl medium for 24h or 48h at 37°C and then treated with different stimulations for indicated time. After 24h or 48h incubation, the absorbance (A) was measured at 570nm by the Universal Microplate Reader (ELx800, BioTek Instruments Inc., Winooski, VT). Percentage of cytotoxicity was determined as follows: percentage of cytotoxicity = [1 − (A570 of test sample)/(A570 of control sample)] × 100%. relative MTT incorporation=A570 of test sample/A570 of control sample × 100%.

Analysis of intracellular Kyn expression by flow cytometry

For intracellular Kyn staining, we used a BD Cytofix/Cytoperm Kit (BD Pharmingen,
San Diego, CA, USA) following the manufacturer's instructions. Intracellular Kyn expression was detected with Kyn monoclonal Abs (Santa Cruz, CA) and FITC conjugate-goat anti-mouse IgG (as a secondary antibody; KeyGen Biotech, Nanjing, China). Stained cells were analyzed by a fluorescence-activated cell sorter (Accuri® C6, Becton Dickinson, Franklin Lakes, NJ, USA).

Measurement of intracellular ROS level

ROS Assay kit purchased from Beyotime Institute of Biotechnology was used according to the manufacturer’s instructions. Cells were cultured in a 12-well plate, treated with various stimulations, and then, the cells were harvested and incubated with 100µM DCFH-DA attenuated with serum-free medium for 20min at 37°C in the dark, washed twice with cold PBS. The fluorescence intensity was measured by fluorescence-activated cell sorter (Accuri® C6, Becton Dickinson, Franklin Lakes, NJ, USA).

Assay of mitochondrial membrane potential (MMP)

HCT-116 and HT-29 were stimulated with 1-L-MT or INCB for 48h. MMP was analyzed by JC-1 staining according to the manufacturer's instructions (MMP assay kit with JC-1, Beyotime Biotechnology, Shanghai, China). The JC-1 dye serves as an indicator of MMP; JC-1 aggregates in mitochondria exhibit red fluorescence, but membrane depolarization prevents aggregation and increases green fluorescence. After incubation, cells were washed once with PBS. The intensity of JC-1 staining was measured by fluorescence microscopy at an excitation wavelength of 505nm with a 534-nm emission filter (Olympus fluorescent microscope, Japan).

RNA extraction and real-time RT-PCR

Total RNA was isolated using the TRizol reagent (Invitrogen) according to the manufacturer’s protocol. The concentration and purity of the extracted RNA were measured with the optical densities at 260 and 280nm. RNA samples were reverse transcribed to cDNA and subjected to quantitative PCR, which was performed with
the Light-Cycler_ 96 Real-Time PCR System (Roche) using AceQ qPCR SYBR Green Master Mix (Vazyme).

EdU cell proliferation assay
HCT116 and HT-29 cells were seeded in 12-well plates at a density of 2 × 10^5 cells per well. After adhesion, cells were stimulated with 1-L-MT or INCB for 24h. Cell proliferation was evaluated by measuring EdU incorporation during DNA synthesis according to the manufacturer’s instructions (Baseclick). EdU incorporation was measured using immunofluorescence (Olympus fluorescent microscope, Japan).

Apoptosis analysis
For fluorescence staining assay, the cells were washed with cold PBS, and incubated with Annexin V-FITC and PI in turns. After different stimulations, HCT-116 and HT-29 were re-suspended cells were stained with AnnexinV-FITC in dark for 15min under 4°C and subsequently treated by PI for 5min under the same conditions. The stained cells were subjected to a flow cytometer (Accuri® C6, Becton Dickinson, Franklin Lakes, NJ, USA) for quantitative analysis. Annexin V+/PI- (early apoptosis) together with Annexin V+/PI + cells (late apoptosis) were deemed as apoptotic portion.

Cell cycle assay
After treatment with 1-L-MT or INCB for 24 or 48h, HCT-116 and HT-29 were harvested, spin down and the resulting pellets were fixed in ice-cold 70% ethanol. Fixed cells were centrifuged, washed and re-suspended in PBS containing RNase A (1 mg/ml), and propidium iodide (PI) was added (1.0mg/ml). PI-stained cells were analyzed by a fluorescence-activated cell sorter (Accuri® C6, Becton Dickinson, Franklin Lakes, NJ, USA), followed by the determination of the percentage of cells in G0/G1, S, and G2/M.

Analysis of cell cycle progression and mitotic index
Cell cycle progression was monitored using DNA flow cytometry. DNA was stained with PI, and mitotic cells were quantified by measuring the expression of a mitosis-specific marker, phospho-histone H3 (p-histone H3). In brief, the cells were trypsinized, washed with cold PBS, fixed with ice-cold 70% ethanol for 16h and immunostained with an anti-p-histone H3 antibody (Cell Signaling Technology) followed by a FITC-conjugated goat anti-rabbit antibody (KeyGEN Biotech). The cells were then stained with 4µg/ml PI in PBS containing 1% Triton X-100 and 0.1 mg/ml RNase A. p-Histone H3 levels and the DNA content of individual cells were analyzed using a fluorescence-activated cell sorting cater-plus flow cytometry (Accuri® C6, Becton Dickinson, Franklin Lakes, NJ, USA).

Transfection of siRNA

AhR, BubR1, IDO and MAD2 siRNA were synthesized by Sango Biotech, and transformed according to the manufacturer’s instruction of Ecfect Transfection reagent (Vazyme). The sequences of siRNA are as following:

BubR1 siRNA, 5’-CACTTATGGTACTGTATAT-3’,
MAD2 siRNA, 5’-CTACTACAATCCACAAA-3’,
AhR siRNA, sense 5’-GAGGCUCAGGUUAUCAGUUUAUCA-3’,
antisense 5’-UGAAUAAACUGUAACCUGAGCCUC-3’
IDO siRNA, 5’-GAACCCACTGCTTACTGGCTT-3’.

Animal studies and colitis-associated colon cancer

Animal welfare and experimental procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, the United States) and the related ethical regulations of our university. Pathogen-free male C57BL/6 mice and Rag1-/- mice were purchased from the Model Animal Research Center of Nanjing University (Nanjing, China) at 5 weeks of age, IDO knock-out (IDO-/-), on the C57BL/6, were originally purchased from The Jackson Laboratory (Bar Harbor, ME). They were exposed to a 12:12-hour light/dark cycle. At 6 weeks of age, mice received intraperitoneal 10 mg/kg azoxymethane (AOM, Sigma, St. Louis,
MO) followed by 7-day cycles of sterile filtered dextran sodium sulfate (DSS, TdB Consultancy, Uppsala, Sweden) at 2.5% in their drinking water. Purified diets were prepared by Xietong-organism Co., LLC (Nanjing Jiangsu, China). Mice were allowed to eat ad libitum. Mice were euthanized at the end of the experiment and colon tissue harvested.

Histopathology

Immunohistochemical stains against IDO, Kyn, CDC20, Ki67 and caspase-3 were performed using immunohistochemistry kit (Key-GEN, Nanjing, China). Briefly, paraffin-embedded slides were deparaffinized, rehydrated and washed in 1% PBS-Tween. Then they were treated with 3% hydrogen peroxide and blocked with 10% goat serum for 1h at 37°C. Slides were incubated with primary antibodies in PBS containing 1% BSA (1:50) for 1h at 37°C. Biotinylated secondary anti-rabbit antibodies were added and incubated at room temperature for 1h. Streptavidin-HRP was added, and after 40min the sections were stained with DAB substrate and counterstained with hematoxylin. Histopathology analysis was performed as previously described. 

Immunofluorescence

Immunofluorescence was performed on paraffin-embedded colonic tissue sections (5 μm). The sections were deparaffinized, rehydrated and washed in 1% PBS-Tween. Then they were treated with 3% hydrogen peroxide, blocked with 10% goat serum and incubated with Tunnel for 1h at 37°C. The slides were stained with DAPI. Images were acquired by confocal laser-scanning microscope (Olympus, Lake Success, NY). Settings for image acquisition were identical for control and experimental tissues.

Statistical analysis

Results are presented as the mean±SD. Statistical analyses were performed with the t-test for two groups or one-way ANOVA (GraphPad Software) for multiple groups. p Values<0.05 were considered significant.
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Conflict of interest statement
The authors declare no conflict of interest.

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**Figure legends**

**Fig.1 IDO inhibitors suppressed the proliferation of colon cancer cells.**

A. B. HCT116 and HT-29 cells were seeded into 96-well plates at a density of $5 \times 10^3$ cells/well and then treated with various concentrations of 1-L-MT for 24h or 48h. Cell viability was determined using MTT assay. C. HCT-116 and HT-29 were treated with 1-L-MT (5mM) and INCB (50µM) for 24h or 48h. Kyn acid secretion was examined by Elisa. D. HCT-116 and HT-29 were stimulated with 1-L-MT or INCB for 48h, and then they were costained with PI and FITC-conjugated Annexin V. The apoptosis of colon cancer cells was detected by flow cytometry. HCT116 and HT-29 were treated with the indicated concentrations of 1-L-MT and INCB for 24h (E) or 48h (F). Cell cycle distribution was measured using flow cytometry. The results are representative of three independent experiments and are expressed as the mean ± SD.
*p < 0.05, **p < 0.01, ***p < 0.001

**Fig. 2** IDO inhibition induced mitotic cell accumulation in colon cancer cells

A. HCT-116 and HT-29 were treated with 1-L-MT (5mM), INCB (50µM) and INF-γ (4ng) for 24h. Cell proliferation (EDU positive) assay was measured using immunofluorescence cytochemistry (×200). The protein levels of BubR1, MAD2, p-Histone and CDC20 were detected by Western blot (B-E). F. Mitotic cells were stained with p-histone H3 and PI, and then analyzed by flow cytometry. Western blot analysis of BubR1 (G) and MAD2 (H) expressions in HCT-116 and HT-29 transfected with different concentrations of BubR1 and MAD2 siRNA. I, J. BubR1 or MAD2 siRNA-transfected HCT-116 and HT-29 were treated with 1-L-MT (5mM), and the ratio of mitotic cells was analyzed by flow cytometry. The results are representative of three independent experiments and are expressed as the mean ± SD. *p < 0.05, **p < 0.01

**Fig. 3** CDC20 was required in mitotic death of colon cancer cells caused by IDO inhibitors

A, B HCT-116 and HT-29 were stimulated with 1-L-MT or INCB for 24h, and the protein levels of Cyclin B1, CDK, p-CDK Thr 161, p-CDK Try 15, soluble, insoluble and total α-tubulin were detected by Western blot. HCT-116 and HT-29 were stimulated with 1-L-MT or INCB for different time, the protein levels of Pro-PARP, cleaved PARP and MPM2 were examined by Western blot (D-G). C. Microtubule polymerization was analyzed by immunofluorescence staining colon cells treated with 1-L-MT, INCB and Paclitaxel for 24h (×600). BubR1 or MAD2 siRNA-transfected HCT-116 and HT-29 were treated with 1-L-MT or INCB, and the protein level of PARP was detected (H, I). J. Western blot analysis of CDC20 expressions in HCT-116 and HT-29 transfected with different concentrations of CDC20 siRNA. K. CDC20 siRNA-transfected HCT-116 and HT-29 were treated with 1-L-MT and INCB. Pro-PARP and cleaved PARP were analyzed by western blot. L, M. The apoptosis of colon cancer cells exposed to those stimulations was detected by flow cytometry. The
results are representative of three independent experiments and are expressed as the mean ± SD. *p < 0.05, **p < 0.01, N.S means no significant difference.

Fig. 4 AhR was dispensable in Kyn-induced CDC20 transcription

A. HCT-116 and HT-29 were stimulated with Kyn, Trp, INF-γ for 24h, and cell viability was measured by MTT. The expressions of cell cycle and apoptosis-related proteins in HCT-116 (B) and HT-29 (C) were determined by Western blot. D. The mRNA level of CDC20 was measured by real-time RT-PCR. E. AhR expression was analyzed by Western blot. F. Western blot analysis of AhR expressions in HCT-116 and HT-29 transfected with different concentrations of AhR siRNA. G. AhR siRNA-transfected HCT-116 and HT-29 were treated with 1-L-MT and INCB, and CDC20 expression was examined by Western blot. H. HCT-116 cells were stimulated with 1-L-MT, INCB and Kyn. AhR nuclear translocation was determined by Western blot. I. CH-223191- pretreated HCT-116 and HT-29 were incubated with 1-L-MT and INCB. CDC20 expression was detected. J. The location of Kyn was analyzed by immunofluorescence cytochemistry (scale bar, 10µm). The results are representative of three independent experiments and are expressed as the mean ± SD. *p < 0.05, **p < 0.01

Fig. 5 1-L-MT prevented colitis-associated tumorigenesis

A. Induction procedure and groups designed for the AOM/DSS model of CAC (n = 14). B. Body weight. C. Survival rate. D. Representative images. E. Number of polyps per mouse. F. H&E staining (scale bar, 100µm). G. Average clinical score of colons. Clinical parameters (weight loss, stool consistency, bleeding) of indicated mice. H. Colon length. The results are representative of at least six independent experiments and are expressed as the mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001

Fig. 6 The effects of 1-L-MT on CRC were dependent on IDO

A. AOM/DSS model of CAC was performed on WT and IDO-/- mice (n=14). Representative images of tumors in the colon section of different groups were shown.
B. Number of polyps per mouse. C. Survival rate. D. H&E staining (scale bar, 100µm).
E. Average Histology score of colons. F. The ratio of Kyn to Trp was measured by Elisa. G. The expressions of IDO and CDC20 were determined by Western blot. H. Expressions of IDO, Kyn and CDC20 were analyzed by immunochemical staining of paraffin-embedded colon sections (scale bar, 100µm). I. The apoptosis of cancer cells in the colon section was determined by TUNEL assay (scale bar, 50 and 10µm). The results are representative of at least three independent experiments and are expressed as the mean ± SD. *p < 0.05, **p < 0.01, N.S means no significant difference

Supplementary Fig. 1 IDO inhibitors suppressed the proliferation of colon cancer cells.
A, B. HCT116 and HT-29 cells were seeded into 96-well plates at a density of 5 × 10^3 cells/well and then treated with various concentrations of INCB for 24h or 48h. Cell viability was determined using MTT assay. C. HCT-116 and HT-29 were treated with 1-L-MT (5mM) and INCB (50µM) for 24h or 48h. The synthesis of Kyn was determined by flow cytometry. D. The proliferation of HCT-116 and HT-29 were examined by Plate clone formation assay (up) and the cells in a clone were shown in the lower image (×200). The results are representative of three independent experiments and are expressed as the mean ± SD. *p < 0.05, **p < 0.01, ***p< 0.001

Supplementary Fig. 2 IDO inhibition resulted in mitochondrial damage and caspase-dependent apoptosis.
A, B. HCT-116 and HT-29 were stimulated with 1-L-MT (5mM) or INCB (50µM) for different time, and the intracellular level of total ROS was measured by flow cytometry. C. Mitochondrial membrane depolarization analysis was performed by detecting the mitochondrial transmembrane potential using JC-1 staining. The released Cytochrome C was measured by flow cytometry (D-F). The protein levels of cleaved caspase-3, 8, 9 and Bcl-2 were examined by Western blot (G-J). K, L CDC20 siRNA-transfected HCT-116 and HT-29 were stimulated with 1-L-MT and INCB of predicted doses, and the expressions of cleaved caspase-3, 8, 9 and Bcl-2 were
determined by Western blot. The results are representative of three independent experiments and are expressed as the mean ± SD. *p < 0.05, **p < 0.01

**Supplementary Fig. 3** Kyn rescued IDO inhibition-induced mitotic death of colon cancer cells.
A, HCT-116 and HT-29 were stimulated with 1-L-MT (5mM), INCB (50µM) or Kyn (50µM) for 24h, Ki67 expression was analyzed by cell immunohistochemistry. B. The viabilities of HCT-116 and HT-29 were determined using MTT assay. C. Cell cycle distribution was measured using flow cytometry. D. The apoptosis of colon cancer cells was detected by flow cytometry. E, F, The expressions of cell cycle and apoptosis-related proteins were detected by Western blot. G. Western blot analysis of IDO expressions in HCT-116 and HT-29 transfected with different concentrations of IDO siRNA. H, I. IDO siRNA-transfected HCT-116 and HT-29 cells were treated with Kyn (50µM). Ki67 expression was analyzed by flow cytometry. The results are representative of three independent experiments and are expressed as the mean ± SD. *p < 0.05, **p < 0.01

**Supplementary Fig.4** 1-L-MT suppressed IDO/Kyn pathway in CRC.
Protein levels of the inflammatory cytokines INF-γ (A, I), Kyn (C, G) and Trp (B, J) in serum and the colon section were determined by Elisa. The ratio of Kyn to Trp in serum (D) and colon (H) section was measured. E. The mRNA expressions of INF-γ, IDO and CDC20 in colonic tissue were determined. F. Expressions of IDO and CDC20 were analyzed by Western blot. K. Expressions of IDO, Kyn, CDC20, Ki67 and cleaved caspase-3 were analyzed by immunohistochemical staining of paraffin-embedded colon sections (scale bar, 100µm). M. The apoptosis of cancer cells in the colon section was determined by TUNEL assay (scale bar, 50 and 10µm). The results are representative of three independent experiments and are expressed as the mean ± SD. *p < 0.05, **p < 0.01

**Supplementary Fig. 5** 1-L-MT prevented CRC and induced tumor apoptosis
independent of effect on adaptive immunity.

A. AOM/DSS model of CAC was performed on Rag1−/− mice (n=14). Representative images of tumors in the colon section of different groups were shown. B. Number of polyps per mouse. C. Survival rate. D. H&E staining (scale bar, 100µm). E. Average Histology score of colons. F. The expressions of IDO and CDC20 were determined by Western blot. G. Expressions of IDO, Kyn and CDC20 were analyzed by immunochemical staining of paraffin-embedded colon sections (scale bar, 100µm). H. The apoptosis of cancer cells in the colon section was determined by TUNEL assay (scale bar, 50 and 10µm). The results are representative of at least three independent experiments and are expressed as the mean ± SD. *p < 0.05, **p < 0.01


IDO inhibition-induced decreased Kyn, suppressed the transcription of CDC20, resulting in G2/M cycle arrest and mitochondria injuries-mediated apoptosis of colon cancer cells.
A

B

C

D

E

F

G

H

I

1226x1579mm (72 x 72 DPI)