Blockade of IDO-kynurenine-AhR pathway promotes cell apoptosis in carboxyamidotriazole-induced tumor cell dormancy-apoptosis oscillation

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Abstract

Background: Some cancer cells may reshape their genetic make-up, adopt a special metabolism mode and undergo dormancy to endure drug attacks. Blocking survival signals in dormant cancer cells that survive a certain anticancer therapy and eradicating them while dormant may help prevent tumor recurrence and metastasis.

Methods: Two colorectal cancer cell lines C26 and HCT116 were treated with carboxyamidotriazole. Sulforhodamine B assay and Ki67 staining were conducted to detect the cells proliferation response. Cell cycle distribution was measured with BrdU staining. Then treated with CAI, DMF, 1-MT or a combination and analyzed the apoptosis. The in vivo anti-tumor effects of each monotherapy or combination therapy were assessed according to their capability to slow tumor growth and extend the life span of tumor-bearing mice.

Results: The colorectal cancer cells slow growth to escape the pressure of the anti-tumor drug CAI. Blocking the IDO-kynurenine-AhR pathway could promote CRC cells apoptosis in CAI-induced tumor cell dormancy-apoptosis oscillation, facilitating their eradication.

Conclusion: The combination of 1-MT or DMF with CAI may prompt dormant cancer cell to enter an apoptotic state, which is triggered by STAT1 nuclear translocation but obscured by the dormancy-permissive metabolic fitness signals when the tumor cells are exposed to CAI alone.

1. Background

With advances in early diagnosis and treatment of tumors, a dramatic increase in disease -free survival can be seen in some patients with cancer, but they still face a high risk of tumor recurrence[1, 2]. Those disease-free cancer patients have been found to have a subset of tumor cells called dormant cells, which is believed to be the main cause of tumor recurrence[3, 4]. Tumor dormancy is a clinical phenomenon that usually occurred after the radical resection of the primary tumor when micro-tumor foci or remnant tumor cells escape treatment or at the earliest stage of tumor development[5, 6]. Dormant tumor cells are extremely slow growing and undetectable over a certain period of time. However, these tumor cells still have the potential to proliferate, and can be active again years or even decades later, leading to tumor recurrence or distant metastasis[7]. When the proliferation and apoptosis of tumor cells keep balanced, the tumor cell population are in a quiescent state. Or when tumor cells are arrested in the G0 phase of the cell cycle without growing or dividing due to environmental stress they might enter dormancy[8, 9]. A dormant subpopulation of tumor cells is usually very small and difficult to detect, however once tumor dormancy is disrupted, the reactivated tumor cells can be highly proliferative and give rise to tumor progression and relapse[10]. Dormancy phenomenon has been found in a variety of tumor[11–13], which is considered to be responsible for multiple malignant manifestations of a tumor including relapse and metastasis, chemotherapy resistance and immune evasion. Therefore, targeting tumor cell dormancy can be a useful approach to fight against cancer.

Carboxyamidotriazole (CAI) is an inhibitor of calcium influx and has shown antiangiogenic, antiproliferative, and antimetastatic properties in preclinical studies[14–17]. In addition, CAI exhibited a protective role in treating cancer-associated cachexia and had good synergistic effect with several first-line anti-cancer drugs[18, 19]. In the early stage of tumorigenesis, CAI exerted a significant anti-cancer effect but in the stage of tumor relapse or progression, the role of CAI was relatively weakened, which can be observed in our animal experiments and previous clinical studies[20, 21]. Recently we found that long-term CAI treatment produces a suppressive effect on $CD8^+$ T cells through activation of IDO-Kyn-AhR cascade, which contributes to a weakened immune response in tumor initiation, growth and metastasis[22]. Providing evidence that the effects that hamper the in vivo anti-tumor capability of CAI might occur through the IDO-Kyn-AhR cascade. The cytostatic rather than cytotoxic properties of CAI exhibited in a variety of tumor cells observations suggest a possibility that CAI might play a role in cancer cell dormancy. Exploring the signaling cascade triggered by CAI in tumor cells and understanding the mechanism that relates to tumor dormancy might point to a more promising anti-cancer strategy.

In this study, we provide evidence that CAI induces CRC cells into dormancy in vitro and in vivo through the IDO1-Kyn-AhR-p21 cascade. Blocking IDO1-Kyn-AhR pathway prompts tumor cells re-enter the cell cycle and increases their susceptibility to CAI. A combined treatment with CAI plus 1-MT (an IDO1 inhibitor) or DMF (an AhR inhibitor) produced significant anti-tumor effects.

2. Materials And Methods

2.1 Animals and Cell lines

Female nude mice, BALB/c mice and NOD-SCID mice, 6–8 weeks old, were purchased from Center of Medical Experimental Animals of Chinese Academy of Medical Science (Beijing, China). These animals were maintained in the Animal Facilities of Chinese Academy of Medical Science under pathogen-free conditions. All studies involving mice were approved by the Animal Care and Use Committee of Chinese Academy of Medical Science.

Mouse tumor cell lines C26 (colon cancer), B16 (melanoma) and human tumor cell lines HCT116 (colon cancer), A375 (melanoma) and MCF-7 (breast cancer) were purchased from China Center for Type Culture Collection (Beijing, China) and cultured in RPMI1640 (Thermo fisher, MA, USA) with 10% fetal bovine serum (FBS) (Gibco, MA, USA), except that MCF-7 cells were grown in DMEM medium (Gibco, MA, USA) with 10% FBS.

2.2 Reagents

Carboxyamidotriazole was synthesized by the Institute of Materia Medica, Chinese Academy of Medical Sciences (Beijing, China).Oxaliplatin, Irinotecan and Paclitaxel were purchased from Selleck Chemicals (Los Angeles, USA). 1-Methyl-L-tryptophan, 3', 4'-Dimethoxyflavone and L-Kynurenine sulfate salt were purchased from Sigma-Aldrich (Saint Louis, USA).

2.3 Cell cycle analysis

Cells were incubated with 50 mM BrdU (BD Bioscience, NJ, USA) for 1 h and cell cycle analysis was performed using BD Pharmingen APC-BrdU Flow Kits according to the manufacturer's protocol (BD Bioscience, NJ, USA). The samples were analysed by flow cytometry on a BD Accuri C6 Flow Cytometer (BD Bioscience). In some experiments, BrdU (1 mg, BD Bioscience, NJ, USA) was i.p. injected into mice 18 h before mice were killed. The primary tumor cells were isolated from tumor or ascites and used for cell cycle analysis.

2.4 Immunohistochemistry Staining

Tumor sections from a nude mice-bearing HCT116 and BALB/c-bearing C26 transplant model were baked at 60 °C for 20 minutes, deparaffinized with xylene, and rehydrated in graded ethanol series.After antigen retrieval and endogenous peroxidase activity blocking, the slides were stained for Ki67(ab16667, dilution 1:250; Abcam, Cambridge, UK). Localization of specific reactivity was detected using a secondary antibody conjugated to peroxidase followed by observation with 3, 39-diaminobenzidine (DAB) substrate (Zhongshan Golden Bridge Biotechnology, Beijing, China). Slides were counterstainedwith hematoxylin.

2.5 RT–PCR

Total RNA was isolated from cells using Pure RNA Extraction Kit (Bio Teke Corporation) and was transcribed to cDNA by using a TransScript First-Strand cDNA Synthesis Supermix (TransGen Biotech Co, Beijing,China). The primer sequences are shown as follows: Murine IDO1, 5'-TGGCGTATGTGTGGAACCG-3' (sense) and 5'-CTCGCAGTAGGGAACAGCAA-3' (anti-sense); AhR, 5'- GCCCTTCCCGC AAGATGTTAT-3' (sense) and 5'-TCAGCAGGGGTGGACTTTAAT – 3' (anti-sense); β-Actin, 5'- CCTGAGGCTCTTTTCCAGCC-3' (sense) and 5'-TAGAGGTCTT TACGGATGTCAACGT – 3' (anti-sense); Homo sapiens IDO1, 5'-AGTCCGTGAGT TTGTCCTTTCAA – 3' (sense) and 5'-TTTCACACAGGCGTCATAAGCT-3' (anti-sense); AhR, 5'- ACATCACCTACGCCAGTCG-3' (sense) and 5'- CGCTTGGAAGG ATTTGACTTGA – 3' (anti-sense); β-Actin, 5'-GAGACCTTCAACACCCCAGC-3' (sense) and 5'- ATGTCACGCACGATTTCCC-3' (anti-sense). Messenger RNA (mRNA) expression quantification was normalized by β-actin mRNA expression. Real-time PCR was performed using ABI stepone plus (Applied Biosystems, MA, USA). Values are mean ± SEM from three independent experiments which were performed in duplicate. Statistical comparisons among groups were performed using ANOVA followed by Fisher's PLSD. Values of all parameters were considered statistically significant difference at a value of $P < 0.05$.

2.6 siRNA transfection

For the adherent siRNA transfections; cells were plated at fifty percent density in 6 well plates. Transfection of siRNA into HCT116 cell lines was carried out using Lipofectamine™ 2000 Transfection Reagent (Invitrogen CA, USA), with either scrambled siRNA or siRNA at a final concentration of 50 nM/well.

2.7 Western blotting

Cell lysate containing 30 μg protein was subjected to SDS/PAGE and separated proteins were transferred onto PVDF membrane. After being blocked with 5% nonfat dry milk in Tris-buffered saline containing Tween-20 the membrane was incubated with the primary antibodies overnight. Antibodies against IDO1,

AhR were purchased from Abcam. Anti-p21, anti-phospho-STAT1, anti-STAT1, anti-caspase3, anti-cleaved caspase3 and anti-β-actin were obtained from Cell Signaling Technology. Subsequently, the membrane was incubated with appropriate secondary antibody and the immunoreactive protein bands were visualized using a chemiluminescence kit (Millipore, MA, USA) followed by ECL-based autoradiography (GE healthcare, UK). Western blots are representative of at least three independent experiments.

2.8 Immunofluorescence

Cells were plated on fibronectin (1 μg/ml)-coated coverslips, fixed in 4% paraformaldehyde and permeabilized with 0.2% Triton X-100. Fixed cells were blocked in 5% BSA and probed with monoclonal anti-AhR, phospho-STAT1, (Abcam, UK), in 5% BSA, followed by Alexa 488-conjugated donkey anti-rabbit IgGs (Invitrogen, CA,USA). After 4, 6-diamidino-2-phenylindole (DAPI) staining, slides were mounted in Fluoromount-G (Solarbio, Beijing, China) and stored at 4 °C in the dark.Confocal microscopy was performed on a Leica laser scanning confocal microscope.

2.9 ChIP-qPCR assay

HCT116 cells treated with 10 μM CAI for 48 h were used in the ChIP assay. The ChIP assay was performed using a ChIP-IT® Express Chromatin Immunoprecipitation Kit (Active Motif, CA, USA) according to the manufacturer's protocol. Antibodies used: anti-AhR (Abcam, Cat. Ab2769), IgG control isotype (Cell Signaling 3900). P21 primer sequence: 5'- AGGTTCAAGCGATTCTCCCACC – 3' (sense) and 5'- GTGGTGGCTTACGCCTGTAATC – 3' (anti-sense). All sequences designed to give amplicons < 200 bp. The purified DNA (IP sample) was amplified by qPCR. Fold Enrichment Method was used to normalize the ChIP-qPCR data.

2.10 Statistical analysis

Data shown are from one representative experiment of multiple independent experiments and are given as mean ± SEM. All experiments were performed at least three times. The statistical significance of differences between two groups was determined by the Student's t tests or One-way ANOVA followed by Dunnett t-test for multiple comparisons. P values less than 0.05 ($P < 0.05$) were considered indicative of significance. The analysis was conducted using the Graphpad 6.0 software. Sample exclusion was never carried out.

3. Results

3.1 CAI induces CRC cells into dormancy in vitro.

CAI has been reported to be one of promising drugs for colitis [17]. To better understand the role of this antitumor drug based on previous research, we tested the effect of CAI on CRC cells in vitro. Both CAI and irinotecan had inhibitory effect on tumor growth, but the difference of growth rate was not significant in CAI withdraw groups with control groups, indicating that CAI treated cells re-growing once inducers of dormancy are removed (Fig. 1A). In addition, even though CAI didn't induce apoptosis in HCT116 or C26 cells, their growth was markedly inhibited (Fig. 1B and C). Dormant tumor cells may reduce their response to chemotherapy drugs. Cells that survived after 48 hours of CAI treatment were cultured and exposed to

three first-line anti-colon cancer drugs oxaliplatin, irinotecan and paclitaxel for another 48 hours. These cells were significantly less sensitive to the three chemotherapeutic drugs compared with those cells without pre-treatment with CAI (Fig. 1D). Cell cycle analysis showed that CAI treatment markedly increased the G0/G1 proportion from 7.58–69.4% in the living tumor cells (Fig. 1E and 1F). In line with cell death resistance and cell cycle arrest, we also found that CAI treatment downregulated the level of ATP (Fig. 1G), suggesting that CAI is capable of inducing dormancy. Moreover, the ratio of phosphorylated ERK (p-ERK) to phosphorylated p38 (p-p38), a cell dormancy marker, was found increased in CAI cultured cells also proved this phenomenon (Fig. 1H). The results suggest that CAI might participate the CRC cells dormancy induction and maintenance in vitro.

3.2 CAI induces CRC cells into dormancy in vivo.

We further verified the above effects of CAI with in vivo xenograft experiments. Nude mice or BALB/C mice were subcutaneously injected with HCT116 cells or C26 cells, respectively, when the tumors size reached 5 × 5 mm, mice were divided into 3 groups (n = 8 per group) and treated with either CAI or the vehicle control PEG400 for 14 days. Interestingly, in CAI treated mice, the tumor growth inhibition effect of the indicated compare with CAI withdraw groups (Fig. 2A).Cell cycle analysis showed that CAI induced significant G0/G1 cell cycle arrest in vivo (Fig. 2C and 2D). The immunohistochemistry of tumor cell proliferating marker Ki67 demonstrated reduced proliferation with CAI treatment (Fig. 2E). The inhibition of cell proliferation was more prominent after two weeks of CAI treatment. Taken together, the results suggest that CAI can induce tumour dormancy in vivo with potential clinical significance.

3.3 CAI-induced CRC cells dormancy is associated with IDO-Kyn-AhR-p21 cascade activation

Next, we explored how CAI induced tumor cells into dormancy. After incubation with CAI for 48 hours, the levels of kynurenine (Kyn), one of the metabolites of essential amino acid tryptophan in the supernatant of HCT116 cells and C26 cells were significantly increased (Fig. 3A). Considering that Indoleamine-2, 3-dioxygenase 1(IDO1) plays a key role in catalyzing tryptophan to Kyn, we further tested the IDO1 mRNA and protein levels in both cell lines. Not surprisingly, CAI treatment upregulated IDO1 expression at both mRNA and protein levels in HCT116 or C26 cells (Fig. 3B and 3C). The results of cell cycle analysis showed that that inhibition of IDO1 blocked the CAI-induced G0/G1 cell cycle arrest (Fig. 3D). Given the induction of Kyn by IDO1, we hypothesized that CAI possibly prompts tumor cells into a quiescent state by increasing the intracellular Kyn level.

How does CAI affect tumor cell proliferation through IDO1-Kyn signaling pathway? It has been reported that the cytoplasmic transcription factor aryl hydrocarbon receptor (AhR), serving as an environmental sensor and cell cycle checkpoint, can be activated by exogenous xenobiotic ligands such as 2,3,7,8-tetrachlorodibenzo-pdioxin[23] or by endogenous metabolites including Kyn[24, 25]. In line with the increased intracellular level of Kyn enhanced transcription activity of AhR in CAI-treated HCT116 was observed as demonstrated by the immunofluorescence assay (Fig. 3E). Besides, CAI markedly upregulates the mRNA and nuclear protein expression of AhR in both HCT116 and C26 cells (Fig. 3F and 3G). To explore the potential mechanisms of cycle arrest by CAI, we conducted a genome-wide transcriptional profile analysis. CAI significantly upregulated expression of p21 without significantly

altering expression of other cycle arrest-related genes (Fig. 3H). Combined with the above results, the protein expression of a cyclin-dependent kinase (CDK) inhibitor p21 was increased by CAI in a time-dependent manner (Fig. 3I). .The results of cell cycle analysis showed that that inhibition of AhR blocked the CAI-induced G0/G1 cell cycle arrest (Fig. 3J). The ChIP-qPCR data further confirmed that the AhR dependent expression of p21 in HCT116 cells in the presence of CAI tremendously enhanced the activity of the p21 transcriptional program (Fig. 3K). These results suggested a potential role for AhR and p21 in cell cycle regulation and cell dormancy direction when tumor cells are exposed to CAI.

3.4 Combining CAI with 1-MT or DMF promotes nuclear translocation of phosphorylated STAT1 and produces synergistic apoptosis-inducing effects in CRC cells

Based on the knowledge that IDO1 is upregulated by IFN-γ-STAT1 signaling [26–28], we speculated that the upregulation of IDO1 by CAI may involve similar STAT1-related mechanism. When HCT116 or C26 cells were exposed to CAI (10 μM) for 48 hours the expression levels of p-STAT1 were significantly upregulated (Fig. 4A). Representative immunofluorescence staining images of HCT116 cells further showed that CAI treatment stimulated both p-STAT1 and AhR to enter the nucleus. When combined with the inhibitor of IDO1–AhR pathway, 1-MT or DMF, the nuclear translocation of STAT1 was enhanced. On the contrary, CAI-induced nuclear translocation of AhR was suppressed (Fig. 4B). Translocation of p-STAT1 into the nucleus may trigger cell apoptosis by activating caspase-3[29]. We analyzed the expression of cleaved caspase-3 in lysates of HCT116 cells which were incubated with the indicated single agent or combined agents for 48 hours. Strong expression of cleaved caspases-3 was observed in combined agents (CAI plus 1-MT or DMF)-treated cells. Quantitative analysis of the expression ratio between cleaved caspse-3 and total caspase-3 in both combination groups was up to 1.4 and 1.7fold (Fig. 4C). To clarify the effects of combining these agents, HCT116 cells were treated with the drugs individually or in combination, and Annexin V/propidium iodide staining was used to evaluate apoptosis. After 48 hours, IDO1 inhibitor 1-MT(0.5 mM), AhR inhibitor DMF (20 μM), and CAI (10 μM) treated HCT116 cells elicited 1.23%, 2.76% and 6.77% apoptosis, respectively, and the combination induced a larger proportion of cell apoptosis (up to 27.6% and 43.2%). In C26 cells, CAI combine with 1-MT/DMF the combination-induced apoptotic cell percentage was 30.5%, and 42.0%, respectively (Fig. 4D). Taken together, these results implied that CAI simultaneously activates STAT1 and IDO-Kyn-AhR-p21 signaling pathways and p21 expression. Blocking the IDO1-Kyn-AhR cascade may lead to CAI induced apoptosis via p-STAT1 nuclear translocation and cleaved caspase 3.

3.5 Combining CAI with 1-MT or DMF synergistically inhibits tumor growth of CRC cells in vivo and improves survival in tumor-bearing mice.

Our in vitro data inspired us to evaluate the efficacy of this combination in colorectal carcinoma-bearing mice. BALB/c mice were subcutaneously injected with 1×10^5 C26 tumor cells. When transplantation tumor reached at 5 mm × 5 mm, the animals were randomly assigned into four groups. The animals were then treated with CAI, or IDO1 (1-MT) or an AhR inhibitor (DMF) or the combination treatment with 1-MT/DMF and CAI for 21 days. The result showed that the combined treatment was superior to each drug

alone by significantly repressing the tumor growth and prolonging the survival (Fig. 5A and 5B). This might be due to an inhibition of Kyn which may generate an antitumor effect through an immune modulation mechanism as our work has illustrated before [30, 31]. To further evaluate the combined therapeutic efficiency, nude mice with HCT116 were treated with CAI, or 1-MT or DMF, or the combination of 1-MT/ DMF and CAI. In this case, the treatment efficacy was also observed with inhibited tumor growth and prolonged survival (Fig. 5C and 5D). In addition to CRC cells, melanoma cells A375 and B16, breast carcinoma cells MCF-7 was also effectively treated by the combination of CAI and 1-MT or DMF which had a larger proportion of cell apoptosis (Fig. 5E-G). Then, we compared the effect of DMF plus CAI in A375 melanoma-bearing mice. The result showed that the combined treatment was superior to each single drug treatment by significantly repressing tumor growth and prolonging the survival (Fig. 5H). These data together suggest that CAI plays an important role in tumor dormancy regulation by activating IDO1-Kyn-AhR pathway and blocking this pathway produces an ideal treatment strategy against cancers by combining with CAI treatment.

3.6 Blocking IDO1-AhR pathway destroys tumor propagation potential of the dormant primary human CRC cells during CAI treatment

Next, we examined whether CAI could also induce dormancy in primary human CRC cells. To this end, the primary CRC cells were isolated from patients, and then treated with CAI. We found that consistent with the observations in HCT116 or C26, CAI also induced primary human CRC cells into dormancy, as evidenced by the upregulated human IDO1, AhR and p-STAT1 (Fig. 6A), and the induction of cell cycle arrest at G0/G1 phase (Fig. 6B and 6C). In addition, we found that the combination of CAI and 1-MT or DMF significantly promoted primary human CRC cells apoptosis (Fig. 6D). Moreover, to support those results, the primary CRC model in NOD-SCID mice was established. Conspicuously, combining CAI and 1-MT or DMF also decreased the tumor size and prolonged the survival (Fig. 6E-H), which is consistent with the above findings.

4. Summary

At present, the treatment methods of tumor mainly include surgery, radiotherapy and chemotherapy. The minimal residual tumor cells that survive these treatments can last for months, years, even decades asymptomatically and may eventually lead to tumor metastases and relapse. Three mechanisms are thought to be involved in this latency state: angiogenic dormancy, immune-mediated dormancy and cellular dormancy[12, 32]. Our findings suggest that CAI involves in cellular dormancy. In the angiogenic dormancy or immune-mediated dormancy state, cell proliferation and apoptosis can reach a dynamic balance, and the tumor won't expand beyond a certain size because of either limitations in blood supply or an active immune system[32, 33]. Although the immune cells can be activated to kill cancer cells, a lot of components in the tumor microenvironment interact with tumor cells and support the tumor cells to survive and invade[34, 35]. Thus, it is important to find efficient strategies to change the dormancy state of the tumor cells and kill them eventually[36, 37].

In this study, cellular dormancy has been described in terms of growth kinetics studying: withdrawing CAI the potential of cell proliferation were restored, after CAI treated the cells resistant to the traditional chemotherapy drugs, tumor cells quiescent in the G0/G1 phase, survival assays and tumor cell biology: cells reduce energy consumption, down regulation of ki67 and a low ERK/p38 signalling ratio (Fig. 1H). Different from static, dormancy is reversible and cells rendered to differentiation in the activation of selective programmes[38]. We provide evidences that CAI can directly target tumor cells and drive them into dormancy through an AhR-mediated pathway, and that blocking IDO1-Kyn-AhR leads to the disruption of CAI-induced dormancy (Fig. 6I) and promote tumor cell apoptosis via p-STAT1 nuclear translocation[29, 39]. The activation of AhR by some of its ligands participates among others in pathways critical to cell cycle regulation, which in turn might lead to dormancy[40]. Through transcriptome microarray assay, we found that CAI treated would increase the expression of p21 in cell cycle related genes significantly (Fig. 3H). Consistent with this observation, AhR knockdown would decrease the cell cycle arrest of CAI effects. Furthermore, ChIP assay indicated that AhR bound to the p21 promoter with CAI treated. Taken together, these results indicate that increase of AhR could induce tumor cells dormancy by up-regulating p21 expression.

In the meantime,the phosphorylated STAT1 also as a mediator of caspase-3 activation[41–43]. Previously, tumor cells were low reacted to CAI-mediated apoptosis despite the STAT1 induced caspase3 actived, whereas 1-MT/DMF inhibited IDO1-Kyn-AhR signaling pathway, this effect could be emerged. In this study, the Western blot results showed that under combination conditions further increased p-stat1 and cleaved caspase 3 protein levels, in keeping with apoptosis results (Fig. 4D).

CAI exposure demonstrated inhibition of in vitro and/or in vivo growth of a variety of tumor cell lines and is capable of regulating the secretion of a variety of cytokines like IFN-γ in T cells. These immuno-modulatory effects are widely accredited for CAI ability to act as an antitumor agent. Currently, T cell and antibody-based immunotherapies have made great success in combating cancer. Some cytokines released by the immune system play key roles in immune surveillance [44]. However, some of them can also induce the dormant state of tumor cells that have not been killed [45, 46].Therefore, removing tumor dormancy and combining tumor immunotherapy may be the key to kill all tumor cells and a reasonable clinical choice for doctors and patients. In the same way, preventive treatment with high efficacy and low toxicity is worth trying.

5. Conclusions

The data in this study clearly show that CAI, by virtue of its activating the IDO-Kyn-AhR cascade and phosphorylation of STAT1, induces dormancy in tumor cells, leading to the discovery of the combination of CAI and an IDO1 or AhR inhibitor for effectively attacking dormant tumor cells. These findings may open a new venue for cancer therapy.

6. Abbreviations

Abbreviation	English Full Name
CRC	colorectal cancer
CAI	Carboxyamidotriazole
IDO1	indoleamine 2, 3-dioxygenase-1
AhR	Aryl hydrocarbon receptor
1-MT	1-Methyl-L-tryptophan
DMF	3′, 4′-Dimethoxyflavone
Kyn	kynurenine
IR	irinotecan
OX	oxaliplatin
PTX	paclitaxel
PI	propidium iodide

Declarations

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None.

Authors' contributions

L.G. and R.J. conceived the project. J.S., L.G., and D. Z participated in the research design. J.S., Q.W. and R.J. conducted the experiments. J.S., and Q.L. contributed new methodology or analytic tools. R.J. and Q.W. provided technical or material support. J.S. and L.G. performed the data analysis. L.G., R.J. and J.S. wrote the manuscript.

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Availability of data and material

All data are available in this article (and its supplementary information files).

Ethics approval and consent to participate

All animal studies and procedures were approved by the Institutional Animal Care and Use Committee of Peking Union Medical College (registration number: ACUC-A02-2017-013).

Consent for publication

Not applicable.

Competing interests

The authors declare no competing financial interests.

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Figures

Figure1

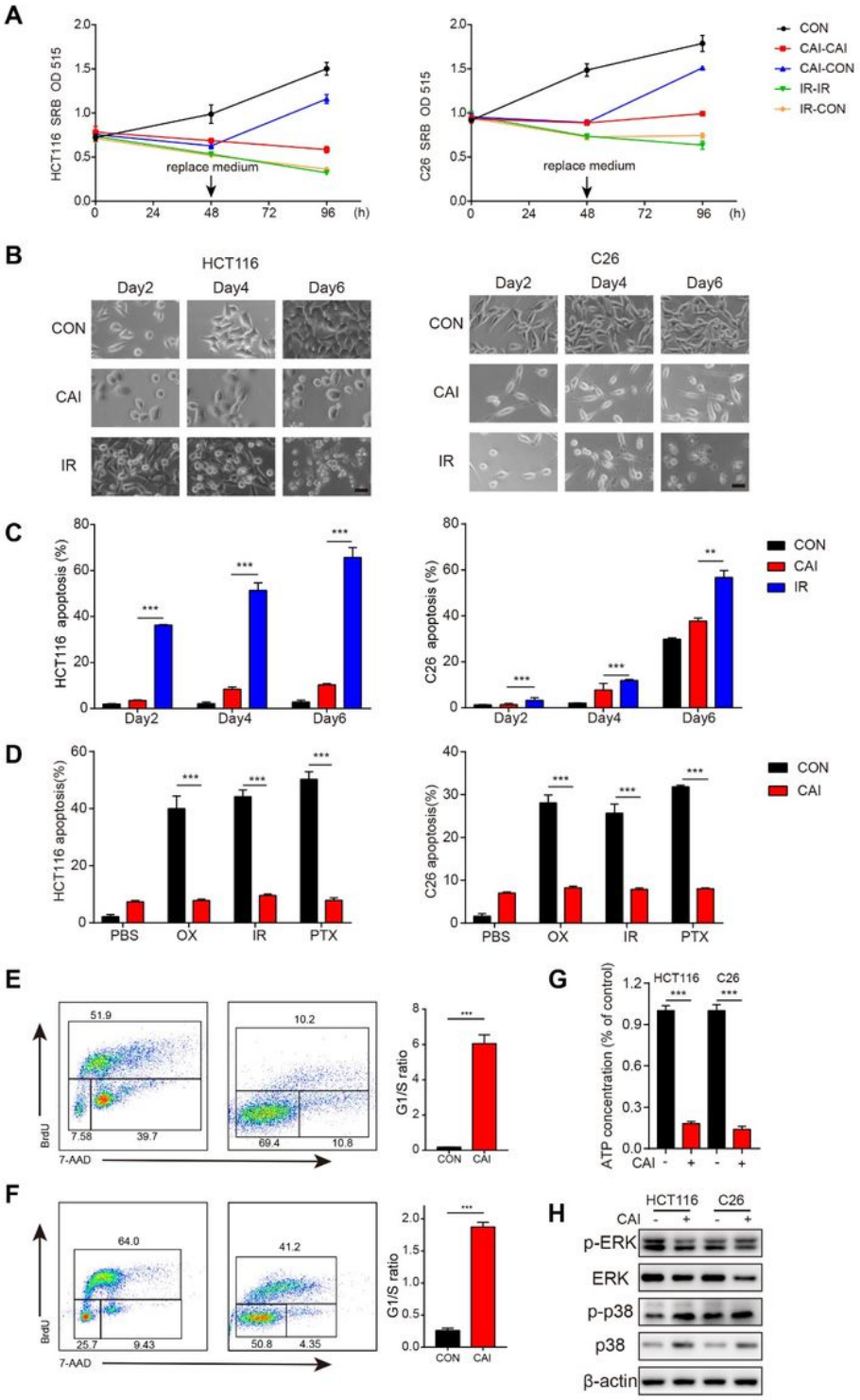

Figure 1

CAI induces CRC cells into dormancy in vitro. (A) Cytostatic activity of CAI on CRC cells as well as the proliferation of surviving cells after drug withdrawal (left: HCT116 cell line; right: C26 cell line). Cells were incubated in medium containing vehicle (CON), CAI (10 μM) or irinotecan (10 μM) for 48 hours, then replace medium with or without the original drug and culture cells for another 48 hours (n = 4). (B and C) HCT116 or C26 cells were treated with DMSO, CAI (10 μM) or irinotecan (10 μM) for 2 days, 4 days or 6

days. (B) Morphology of cells under microscopy, Bar, 50 μm.and (C) The percentage of apoptotic cells was measured by flow cytometry. (D) The tumor cells without any treatment (CON) or those adherent surviving cells after 10 μM CAI treatment for 48 hours were collected, grouped and treated with Oxaliplatin (OX, 1μM) or Irinotecan (IR, 10μM) or Paclitaxel (PTX, 5nM) separately for 48h. Cell apoptosis was measured by flow cytometry. (E and F) Representative flow cytometric plots or histograms of cell cycle analysis. HCT116 cells (E) or C26 cells (F) were treated with DMSO or CAI (10 μM) for 48 hours, then were harvested and stained with BrdU and 7-AAD. (G) ATP concentration in HCT116 and C26 cells with or without 48 h of CAI (10 μM) treatment. (H) The expression of p-ERK and p-p38 in HCT116 and C26 cells in the absence or presence of CAI was determined by western blot. Data shown are representative of three independent experiments and error bars represent mean ± SEM. ***P<0.001, **P<0.01, (Student's t-test).

Figure2

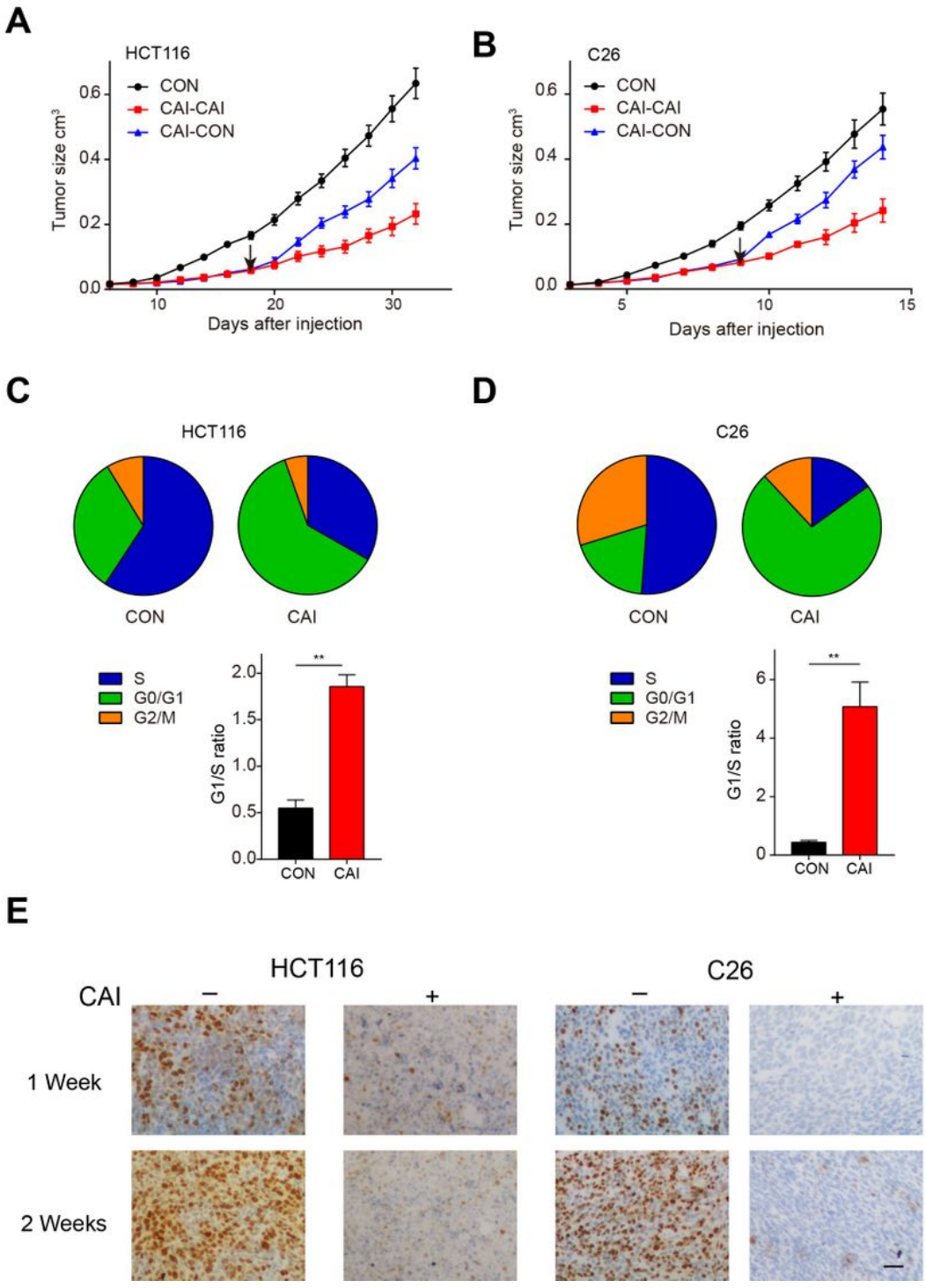

Figure 2

CAI induces CRC cells into dormancy in vivo. The mice bearing 5×5 mm HCT116 or C26 were treated with PBS, CAI (20 mg/kg), for 14 days. Seven days after drug administration, some of the CAI-treated mice initiated CAI withdrawal pattern. (A and B) Tumor growth curves. (C and D) Nude mice or BALB/c mice were subcutaneously injected with 2×105 tumor cells (HCT116 or C26 cells, n = 6 in every group) and received CAI (20mg/kg) for 7 days from the day when tumors reached 5 mm in diameter. Primary tumor

cells were isolated and applied to cell cycle analysis. (E) IHC staining in HCT116 or C26 tumor xenografts to detect Ki-67 expression. Scale bars represent 50 mm. Data shown are representative of three independent experiments and error bars represent mean ± SEM. **P<0.01,(Student's t-test).

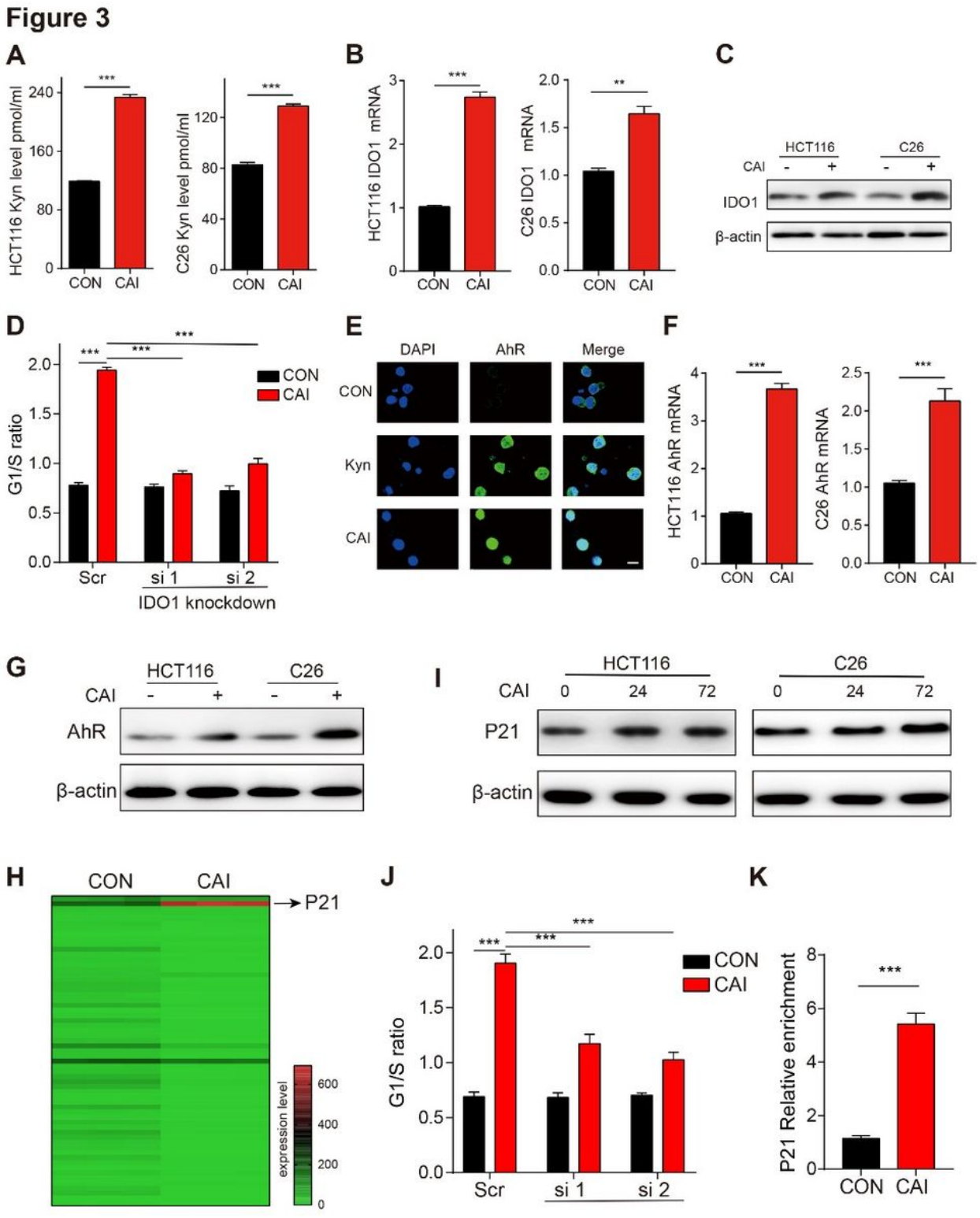

Figure 3

CAI-induced CRC cells dormancy is associated with IDO-Kyn-AhR-p21 cascade activation. HCT116 or C26 cells were incubated with or without 10 μM CAI for 48 hours. (A) The Kyn levels in cell lysate were

determined. (B and C) The expression of IDO1 in HCT116 or C26 cells was determined by real-time PCR (B) and western blotting (C). (D) Scramble siRNA (Scr) or siRNAs against IDO1 was delivered to HCT116 cells. siRNA-mediated IDO1 knockdown in HCT116 cells diminishes CAI induced G0/G1 cell cycle arrest. (E) HCT116 were treated with 200 mM Kyn or 10 μM CAI for 2 days, both CAI and Kyn promoted the translocation of AhR from the cytosol to nucleus by immunostaining assay. Bar, 10 μm. (F and G) The mRNA and protein expressions of AhR in HCT116 or C26 cells was analyzed by RT-PCR and Western blotting. (H) Heatmap of cell cycle- related genes in HCT-116 cells treated with or without CAI. (I) HCT116 or C26 cells were incubated with CAI (10 μM) for 0, 24, 72 h, the expression of p21 was determined by western blot. (J) HCT116 cells exposed to CAI (10 μM) or not were transfected with scramble siRNA (Scr) or two different siRNAs against AhR and cell cycle analysis was performed 48 h post-transfection. (K) ChIP-qPCR analysis of AhR-dependent p21 expression in HCT116 cells with or without CAI treatment. ChIP enrichment ratio relative to control is shown. **p<0.01, ***P<0.001. The data are from 3 independent experiments. Graphs represent mean ± SEM.

Figure 4

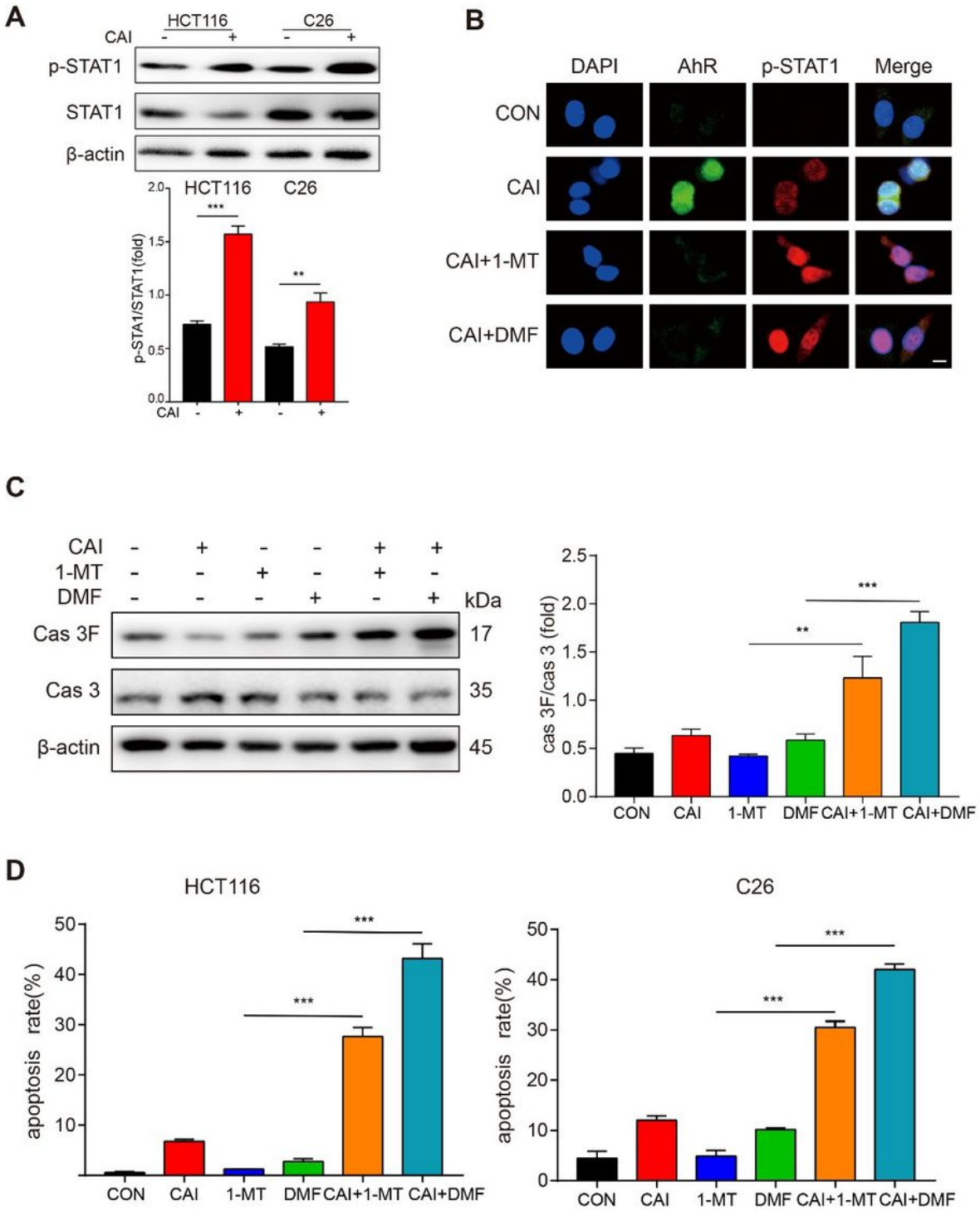

Figure 4

Combining CAI with 1-MT or DMF promotes nuclear translocation of phosphorylated STAT1 and produces synergistic apoptosis-inducing effects in CRC cells. HCT116 or C26 cultured with CAI (10 μM)、1-MT (0.2 mM), DMF (20 μM) or the combination of CAI and DMF or CAI and 1-MT for 48 h. (A) Western blot images showing protein expression of STAT1 and phosphorylated STAT1 in HCT116 or C26 cells treated with or without CAI. (B) Immunostaining for p-STAT1 (red), AhR (green) in DAPI (blue) stained

nuclei of HCT116 cells exposed to various treatments (indicated) are shown. Bar, 10 mm. (C) Protein expression of caspase 3 (Cas 3) and its cleaved form (Cas 3F) in HCT116 cells treated with various single drugs or combinations for 48h were detected by western blot. (D) HCT116 cells and C26 cells were treated with the indicated drugs for 48h. Fraction of cells undergoing apoptosis was analyzed by flow cytometry. The data represent mean ± SEM. (analysis of Student's t-test).

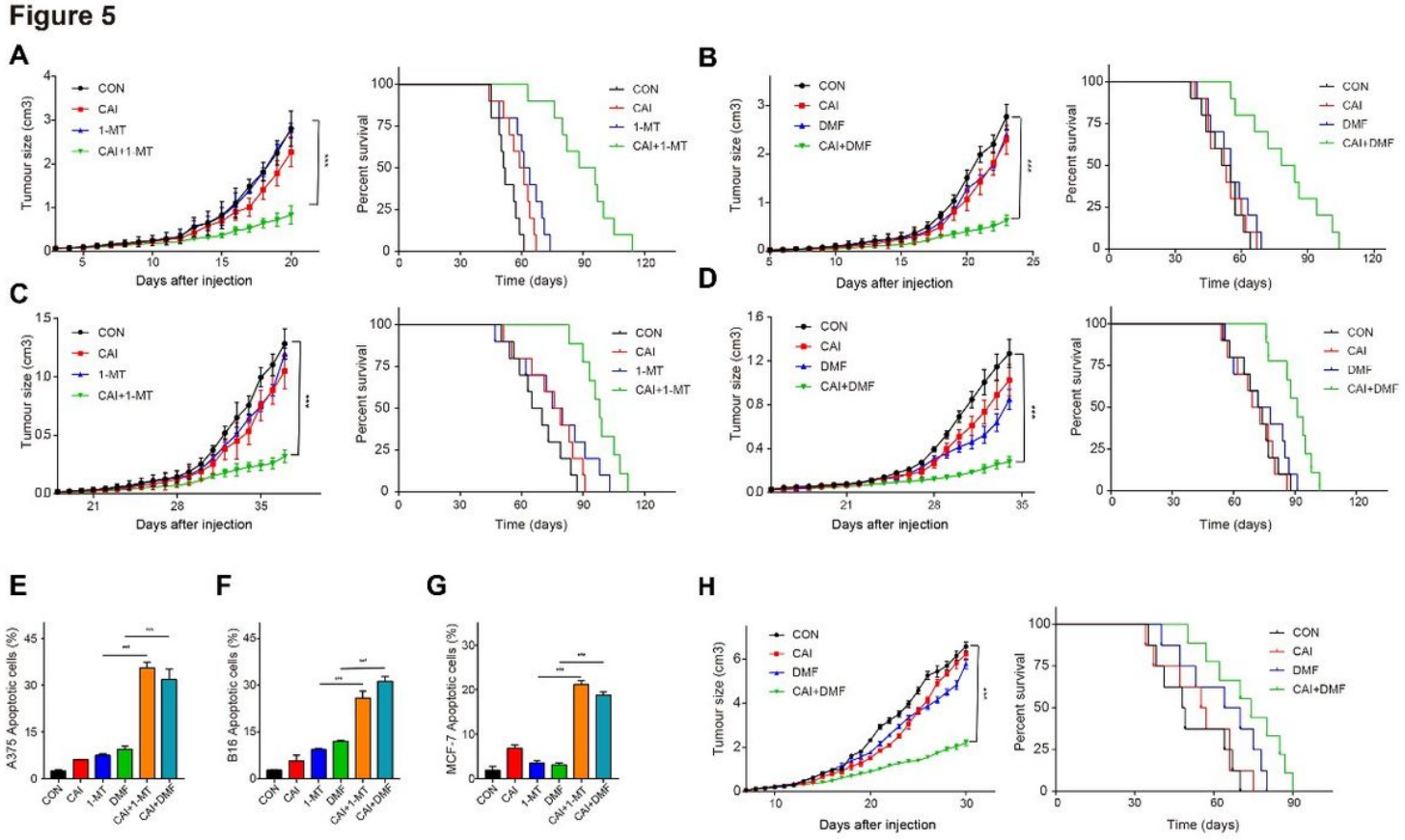

Figure 5

Combining CAI with 1-MT or DMF synergistically inhibits tumor growth of CRC cells in vivo and improves survival in tumor-bearing mice. BALB/c mice or nude mice were subcutaneously injected with (A and B) C26 (1×105 cells /mouse) or (C and D) HCT116 (2×105 cells /mouse). When tumor size was 5×5 mm, mice were treated with CAI, 1-MT, CAI/1-MT, or CAI, DMF, CAI/DMF. The tumor growth (left) was measured and long term survival (right) was analyzed. (E ~G) A375, B16, MCF-7 cells were incubated with CAI (10 μM)、1-MT (0.2 mM), DMF (20 μM) or the combination of CAI and 1-MT or CAI and 1-MT for 48 h. Tumor cell apoptosis was analyzed by flow cytometry. (H)A375 cells (1×105 cells /mouse) were s.c. injected to the NOD-SCID mice (n=8 per group). The tumor growth was measured (left) and long-term survival was analysed (right). The data represent mean ± SEM. ***p<0.001, by one-way ANOVA (A-F, left panels) and Kaplan-Meier survival analysis (a-d, h right panels).

Figure 6

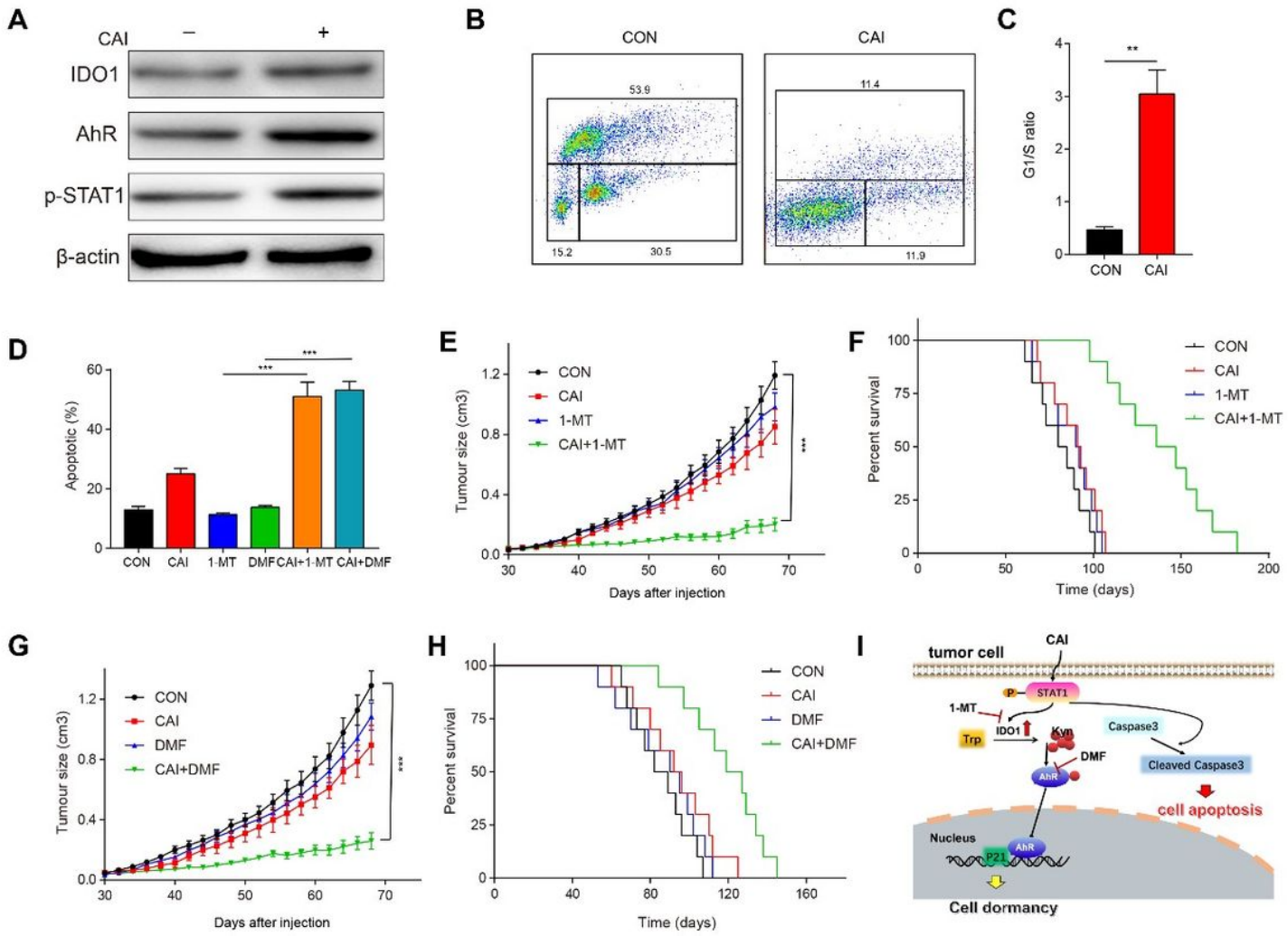

Figure 6

Blocking IDO1-AhR pathway destroys tumor propagation potential of the dormant primary human CRC cells during CAI treatment. (A) Primary human CRC cells were incubated with CAI (10 μM) for 2 days and then subjected to western blotting to detect the expression of IDO1, AhR, phospho-STAT1. (B and C) Cell cycle analysis of the primary human CRC cells treated with CAI for 2 days. (D) Primary human CRC cells were treated with CAI (10 μM)、1-MT (0.2 mM), DMF (20 μM) or the combination of CAI and DMF or CAI and 1-MT for 48 h. Apoptosis was analyzed by flow cytometry. (E ~ H) NOD-SCID mice with 5×5 mm primary human CRC (n=8 per group) were treated with CAI (intragastric injection of 20mg/kg/day), 1-MT (5 mg/ml in drinking water, 3-4 ml/mouse/day) and CAI+1-MT or CAI, DMF (intragastric injection of 10 mg/kg, once every 2 days) and CAI + DMF for 38 days. Tumor size was measured and long-term survival was analyzed. **p < 0.01、***p < 0.001, by Student's T test. The data represent mean ± SEM. (I) Schematic diagram illustrating that CAI activates p-STAT1 and makes tumor cells oscillate between two states of dormancy and apoptosis. Blocking IDO1- Kyn-AhR pathway with 1-MT or DMF might force dormant tumor cells switch to apoptosis.