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Berberine regulates AMP-activated protein kinase signaling pathways and inhibits colon tumorigenesis in mice

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Abstract

Colorectal cancer, a leading cause of cancer death, has been linked to inflammation and obesity. Berberine, an isoquinoline alkaloid, possesses anti-inflammatory, anti-diabetes and anti-tumor properties. In the azoxymethane initiated and dextran sulfate sodium (AOM/DSS) promoted colorectal carcinogenesis mouse model, berberine treated mice showed a 60% reduction in tumor number ($P=0.009$), a 48% reduction in tumors <2 mm, ($P=0.05$); 94% reduction in tumors 2-4 mm, ($P=0.001$) and 100% reduction in tumors >4 mm ($P=0.02$) compared to vehicle treated mice. Berberine also decreased AOM/DSS induced Ki-67 and COX-2 expression. *In vitro* analysis showed that in addition to its anti-proliferation activity, berberine also induced apoptosis in colorectal cancer cell lines. Berberine activated AMP-activated protein kinase (AMPK), a major regulator of metabolic pathways, and inhibited mammalian target of rapamycin (mTOR), a downstream target of AMPK. Furthermore, 4E-binding protein-1 and p70 ribosomal S6 kinases, downstream targets of mTOR, were down regulated by berberine treatment. Berberine did not affect Liver kinase B1 (LKB1) activity or the mitogen-activated protein kinase pathway. Berberine inhibited Nuclear Factor kappa-B (NF- κ B) activity, reduced the expression of cyclin D1 and survivin, induced phosphorylation of p53 and increased caspase-3 cleavage *in vitro*. Berberine inhibition of mTOR activity and p53 phosphorylation was found to be AMPK dependent, while inhibition NF- κ B was AMPK independent. *In vivo*, berberine also activated AMPK, inhibited mTOR and p65 phosphorylation and activated caspase-3 cleavage. Our data suggests that berberine suppresses colon epithelial proliferation and tumorigenesis via AMPK dependent inhibition of mTOR activity and AMPK independent inhibition of NF- κ B.

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Keywords

Berberine; Colorectal cancer treatment; AMP-activated protein kinase; Mammalian target of rapamycin; Proliferation; Prevention

Introduction

Colorectal cancer (CRC) is the third leading cause of cancer-related mortality in the U.S [1]. Inflammatory bowel diseases including ulcerative colitis (UC) and Crohn's disease (CD), correlate with a high risk of developing colorectal cancer [2]. Current conventional chemotherapeutic methods for CRC treatment can have strong side effects affecting the quality of life of patients prompting the need to find new effective therapeutics with fewer side effects. Herbal derived medicines, such as, berberine, may serve as promising therapies for the treatment of CRC.

AMP-activated protein kinase (AMPK) has emerged as a promising cancer related target. AMPK is a key regulator of metabolism and can negatively regulate tumor proliferation. Activation of AMPK is thought to require phosphorylation of AMPK at Thr172 [3]. AMPK can regulate glucose, lipid and protein metabolism in response to changes in fuel availability, oxidative stress, heat shock and hormones [4]. The AMPK pathway can regulate tumor growth and proliferation through its negative regulation of the mammalian target of rapamycin (mTOR) pathway [5]. mTOR is frequently activated in cancer and its activation results in the phosphorylation of the serine/threonine kinase p70S6k and the translational repressor eukaryotic initiation factor 4E binding protein (4EBP1), both of which are important regulators of cell growth, proliferation and protein synthesis [6].

Berberine, a natural compound extracted from the herbal plant *Rizoma Coptidis*, has been used in Chinese medicine for many years. Berberine has anti-diarrheic, anti-inflammation, anti-microbial [7], and anti-tumor properties [7-10]. Berberine has shown efficacy in treating diabetic patients [11]. In rodent models berberine activates AMPK and improves insulin sensitivity [12]. The anti-diabetic drug metformin, which also activates AMPK, is currently being assessed as a therapeutic agent for cancer prevention, including colorectal cancer [13]. The anti-tumor roles of metformin are thought to be mainly due to its activation of the AMPK pathway [14,15]. While it has been shown that berberine can suppress colon tumor growth in a mouse xenograft model [16], the effects of berberine on tumor promotion and progression of CRC have not been determined and the mechanisms of berberine's anti-tumor activity are unclear. We hypothesized that berberine would have chemopreventive and tumor suppressing effects on colorectal carcinogenesis by activating AMPK.

We show both *in vitro* and in a mouse model of colon carcinogenesis that berberine activates AMPK during the early stages of tumorigenesis inhibiting late stage mTOR and inhibits Nuclear Factor kappaB (NF- κ B) activation, leading to inhibition of colon tumorigenesis.

Materials and Methods

Reagents

Berberine (>90% purity by high-performance liquid chromatography, with chemical structure as shown in Supplementary Figure 1), was obtained from National Institutes of Food and Drug Control, China. RPMI-1640, Dulbecco's Modified Eagle's Medium (DMEM) and 0.25% Trypsin-EDTA were purchased from Gibco (Life Technologies, Grand Island, NY, USA). Fetal Bovine Serum (FBS) was obtained from Atlantic Biological. TACS MTT Cell Proliferation Assay kits were ordered from Trevigen (Helgerman, Gaithersburg, MD, USA). Azoxymethane was obtained from Sigma Aldrich (St. Louis, MO, USA). Dextran Sodium Sulfate, 36 000 to 50 000 kDa, was obtained from MP Biomedicals LL, (Solon, OH, USA). NE-PER Nuclear and Cytoplasmic Extraction Kit was ordered from Thermo Scientific (Rockford, IL, USA). Annexin V-FITC Apoptosis Detection Kit I was ordered from BD Biosciences (San Diego, CA, USA).

Antibodies

Anti-phospho-AMPK (Thr172), anti-AMPK, anti-phospho-Acetyl-CoA Carboxylase (Ser79), anti-Acetyl-CoA Carboxylase, anti-phospho-mTOR, anti-mTOR, anti-phospho-p70s6K, anti-p70s6K, anti-phospho-4E-BP1, anti-4E-BP1, anti-COX-2, anti-cleaved-caspase-3, anti-cleaved-PARP, anti-phospho-p53 antibodies, anti NF- κ B kit, anti-phospho-LKB1, anti-LKB1, anti-phospho-AKT, anti-AKT, anti-phospho-ERK1/2, anti-ERK1/2, anti-rabbit and anti-mouse antibodies were from Cell Signaling Technology (Danvers, MA, USA); anti- β -actin was from Sigma-Aldrich (St. Louis, MO, USA); anti-Ki-67 and anti-CD-45 were from Abcam (San Francisco, CA, USA).

Animal Studies (AOM/DSS Induced Carcinogenesis)

All animal studies were conducted in accordance with Frederick National Laboratory of Cancer Research (FNLCR) guidelines for care and use of laboratory animals. Female FVB mice (6-week-old) were purchased from Charles River (Frederick, MD). Animal Diets were from Teklad Lab. After one week accustomed to the new environment and controlled diet (AIN93G), all the mice were randomly divided into 4 groups; the AOM/DSS treated groups, treated with PBS (n=15) or berberine (n=15) and the control groups (no AOM/DSS) treated with PBS (n=5) or berberine (n=5). For AOM/DSS treatment (Supplementary Figure 2), mice were given a single intraperitoneal injection of 10 mg/kg Azoxymethane. One week later the mice were given 2% Dextran Sodium Sulfate (m.w. 36–50 kDa; MP Biomedicals) in drinking water for 7 days. Berberine treatment began 1 day after DSS treatment ended and mice were returned to normal drinking water. Mice were treated with berberine (40 mg/kg in PBS) or PBS by oral gavage, 5 times a week for two continuous weeks (days 15-19 and 23-27), followed by 3 times a week (days 30-70) until to the end of the study. On day 28 after AOM injection, 5 mice of each group were euthanized and the colon tissues were collected for H&E and immunohistochemistry analysis. Liver samples were also collected for Western blotting analysis. The remaining mice of each group (n=10) were euthanized 10 weeks post AOM injection. Colon tissues were collected and the tumor numbers and size were determined. Colon tissue was evaluated by H&E and immunohistochemistry analysis. For the control (no AOM/DSS), mice were administered

berberine (40 mg/kg) or PBS 5 times a week for two weeks. After two weeks (day 28) the mice were euthanized and the liver samples were collected for Western blotting analysis and the colon tissue was collected for H&E and immunohistochemistry analysis.

Histopathology

Colon tissue was harvested from mice and fixed in 4% formaldehyde overnight and stored in 70% ethanol. Fixed sections of colonic tissues were embedded in paraffin, cut into 6- μ m sections and put onto microscopic slides by Histoserv Inc. (Germantown, MD, USA). Slides were stained with hematoxylin–eosin (H&E) for histological analysis. Histology of the colon was evaluated by a board certified pathologist (MSM). Colon tumor formation was diagnosed applying the criteria of a consensus report on murine colon tumors [17] (none, mild, modest, severe). Erosion was only diagnosed if an underlying inflammation was present. Histopathology diagnosis criteria and grading scheme as shown in Saud et al. [18].

Immunohistochemistry

Colonic tissue was harvested from mice, fixed in 4% formaldehyde overnight and stored in 70% ethanol; 5- μ m-thick paraffin embedded sections on microscopic slides were obtained from Histoserv Inc. (Germantown, MD, USA). Slides were deparaffinized in xylene and rehydrated in graded ethanol. The sections were subject to Citrate (pH 6) antigen retrieval in pressure chamber. The sections were pre-incubated in 3% H₂O₂ for 20 minutes and incubated in normal goat serum for 1 hour. Sections were incubated overnight in primary antibody solution diluted as follows: Ki-67, CD45, 1:100; p-mTOR, p-AMPK, and COX-2, 1:500; Cleaved caspase-3, 1: 200; p-p65, 1:50. Slides were incubated in a biotin-conjugated secondary antibody, 1:200 dilutions. The sections were treated with ABC reagent (Vectorstain ABC kit-PK-4000, Vector labs, CA, USA), detected with Diaminobenzidine (Sigma, St. Louis, Missouri, USA) and counterstained in Hematoxylin. Analysis of cells positive for Ki-67 was performed on digitized images using image analysis software ImageProPlus 4.5 (Diagnostic Instruments, USA). Representative images were captured from 5 independently samples.

Cell Culture

HCT116, SW480 and LOVO colon cancer cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and Division of Cancer Treatment and Diagnosis (DCTD) Tumor Repository (National Cancer Institute, USA). HCT116 and LOVO cells were cultured in RPMI1640 supplemented with 10% FBS, 1% penicillin streptomycin and 1% L-Glutamine. SW480 cells were cultured in DMEM supplemented with 10% FBS, 1% penicillin streptomycin and 1% L-Glutamine. Cultures were maintained in a humidified incubator at 37°C in 5% CO₂. A 100 mmol/L stock solution of berberine was prepared in DMSO and diluted as needed into cell culture medium, with a final DMSO concentration 0.1%.

Cell Proliferation Assay

Cells were seeded at a density of 2×10^4 cells per well in a flat-bottomed 96-well plate and cultured for 24 hours. Cells were treated with berberine (0–100 μ mol/L) or 0.1% DMSO for

24, 48 and 72 hours. Subsequently, 10 μ l MTT reagent was added into each well and incubated for another 4 hours until purple dye is visible. 100 μ l detergent reagent was added and the plate was incubated at RT in the dark for 2-4 hours. Absorbance was measured at 570 nm with BIO-RAD Model 680 Micro plate reader. The growth of the berberine treated cells was compared with DMSO-treated control cells. The experiments were independently performed and repeated three times.

Cell Apoptosis Assay

HCT116 and SW480 cells were seeded in 100 mm dishes at a density of 2×10^6 cells per dish in growth medium and grown overnight at 37°C in a humidified incubator with 5% CO₂. Cells were treated with berberine (0-60 μ mol/L) or DMSO (0.1%) for 24 hours. Apoptosis was determined with the Annexin V-FITC Apoptosis Detection Kit I (BD Biosciences, San Diego, CA, USA). The experiments were independently performed and repeated three times.

Western Blot Analysis

Colon cancer cells were seeded on 100 mm plates and treated with either berberine (0 μ mol/L-60 μ mol/L) or DMSO (0.1%) for 24 or 48 hours. The media was removed and cells were washed with cold PBS on ice. Whole-cell extracts were prepared in RIPA lysis buffer (Thermo scientific, Rockford, IL, USA) supplement with proteinase inhibitors (Roche, Mannheim, Germany). Protein concentration was determined by Micro BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA). 30 μ g total protein was denatured, heated for 5 minutes and fractionated on a 10% SDS-PAGE. Proteins were transferred to Nitrocellulose membrane (Bio-Rad) for 2 hours at 300 mA, blocked and incubated with a primary specific antibody in 5% of non-fat milk for 1 hour at room temperature or overnight at 4°C. After washing, blots were incubated with secondary horse radish peroxidase (HRP)-conjugated anti-mouse (1:5000) or anti-rabbit (1:5000) at RT for 1 hour. Antigens were detected with Super Signal West Femto Maximum Sensitivity Substrate (Thermo Scientific, Rockford, IL, USA) exposure on X-ray film. The band intensities were quantified by optical densitometry with Image J software. The experiments were independently performed and repeated three times.

Soft Agar Assay

HCT116 cells were trypsinized and resuspended at 6000 cells in $2 \times$ RPMI media. Cells were treated with DMSO (0.1%) vehicle, berberine (30 μ mol/L), Compound C (10 μ mol/L), or berberine plus Compound C. The bottom layer consisted of 2 ml of 1.2% agarose. The cell suspensions were mixed 1:1 with 0.5% agarose (2 ml/well for a 6 well plate) and layered on top. The cells were maintained in an incubator for 10 days after which the colonies were stained with Nitrotetrazolium Blue chloride (NBT), scanned and counted with GelCount (Oxford Optronix Ltd, Oxford, United Kingdom). The experiments were independently performed and repeated three times.

Statistical Analyses

All data is presented as the means \pm standard deviation. The significance of the difference between groups was evaluated by one-way repeated-measures analysis of variance

(ANOVA) and multiple comparisons with Prism 5.0 software. Student's t-test for paired observation was used where appropriate. $P < 0.05$ were considered to be statistically significant.

Results

Berberine Decreases Tumorigenesis in AOM/DSS Induced Colon Carcinogenesis

In clinical trials, berberine at 300 to 500 mg/kg has been shown to have similar hypoglycemic effects as the anti-diabetic drug metformin [19] and metformin has been shown to suppress colorectal aberrant crypt foci [20]. To determine the efficacy of berberine on inhibiting colorectal cancer, we treated mice with AOM to initiate tumorigenesis followed by DSS to promote tumorigenesis [21,22]. One day after exposure to AOM/DSS mice were treated for 10 weeks with PBS or berberine (40 mg/kg, a dose previously shown to prevent inflammation [23]) (Supplementary Figure 2). Berberine treated mice showed a 60% reduction ($P = 0.009$) in tumor number compared to the PBS treated group. Furthermore, the tumors that developed in the berberine treated mice were significantly smaller compared to the tumors in the control group (Table 1). Histopathological analysis of the tumors revealed that mice in both groups developed multifocal tubular colon adenomas with focal high grade dysplasia. Unlike previously described studies [21,22], tumors in this study were premalignant and did not develop into adenocarcinomas. In addition, the length of colons after AOM/DSS treatment, a marker for evaluating colitis associated loss of elasticity, showed that the colons from the berberine treated mice were significantly longer than the colons from PBS treated mice (Table 1).

Berberine Inhibits AOM/DSS-induced Inflammation and Proliferation

One cycle of DSS exposure is sufficient to produce an inflammatory response in FVB mice [22]. Early onset of DSS-induced inflammation was inhibited by berberine as shown by CD-45 expression, a receptor-linked protein tyrosine phosphatase expressed on leukocytes (Figure 1A). COX-2 is up-regulated in a variety of malignancies and has been linked to the process of tumorigenesis, mainly due to its role in the production of pro-inflammatory prostanoids during the late stages of tumorigenesis [24,25]. We found COX-2 expression to be significantly decreased in berberine treated animals on day 70 (Figure 1C).

Tumorigenesis induced by AOM/DSS results in aberrant proliferation in colon crypts [26]. On day 70 the AOM/DSS treated mice showed increased proliferation in the crypts as detected by Ki-67 immunohistochemistry (IHC) staining (Figure 1B). Berberine suppressed expression of epithelial proliferation marker Ki-67 after AOM/DSS exposure by 60% compared to the PBS control (Figure 1D). In the berberine treated mice, Ki-67 expression was primarily in the basal portion of the colon crypt that contains the multipotent stem cells. Interestingly, berberine had no effect on AOM/DSS induced activation of β -catenin (data not shown), suggesting that berberine inhibition of aberrant proliferation in the crypts occurs downstream of activated β -catenin. Taken together, berberine inhibits AOM/DSS-induced inflammation and proliferation, preventing tumorigenesis.

Berberine Inhibits Growth and Induces Apoptosis of CRC Cells *In Vitro*

In order to elicit possible chemopreventive mechanisms of berberine, we treated three colon cancer cell lines with varied doses of berberine (0-100 $\mu\text{mol/L}$) for 24, 48 and 72 hours. Berberine treatment caused a concentration and time-dependent inhibition of cell growth in HCT116, SW480 and LOVO colorectal carcinoma cells (Supplementary Figure 3A, B and C). Berberine did not inhibit proliferation in the non-tumor cell line HEK293 (data not shown). These results suggest that berberine has strong effect on colon cancer cell proliferation.

To determine the role of programmed cell death on the growth inhibitory effects of berberine, HCT116 and SW480 cells were treated with berberine and followed by staining with FITC Annexin V staining kit. We found that berberine treatment resulted in a dose dependent increase the number of apoptotic HCT116 and SW480 cells (Supplementary Figure 3D, E). These results suggest that the inhibition of CRC cell number by berberine is at least in part due to an increase in programmed cell death.

Berberine Activates AMPK and Acetyl-CoA Carboxylase (ACC) in CRC Cells

Studies have shown that berberine has beneficial effects in the treatment of diabetes and obesity and that these beneficial effects are at least in part mediated by AMPK activity [27]. To determine if berberine inhibition of colon carcinogenesis is linked to the metabolic signal pathway of AMPK, we measured the effects of berberine (0, 15, 30 and 60 $\mu\text{mol/L}$) on AMPK activation in 3 CRC cell lines. Berberine increased phosphorylation of AMPK at Thr172, which reflects AMPK activation, at 24 hours in HCT116 and at 24 and 48 hours in SW480 and LOVO cells (Figure 2A, B and C). HCT116 cells treated with berberine for 48 hours showed significant loss of attachment to the dish. The 15 $\mu\text{mol/L}$ treatment was able to activate AMPK in all three lines, with LOVO cells showing the least activity. Phosphorylation of acetyl-CoA carboxylase (ACC), a well-established AMPK substrate and indicator of AMPK enzyme activity, was increased in berberine treated cells at similar doses (Figure 2A, B and C).

In a time-dependence experiment HCT116 and LOVO cells were treated with 30 $\mu\text{mol/L}$ berberine from 0 to 24 hours. AMPK phosphorylation was detected as early as 30 min after berberine treatment (Figure 2D, E) and continued to increase up to 24 hours. Consistent with activation of AMPK, ACC phosphorylation increased over time, but lagged behind AMPK phosphorylation (Figure 2E). The immediate activation of AMPK by berberine suggests direct interaction with the AMPK signaling pathway.

Activation of AMPK by Berberine is Independent of LKB1 in CRC Cells

Liver Kinase B1 (LKB1) is the primary upstream kinase that phosphorylates Thr172 on AMPK [28-30]. To determine if berberine activation of AMPK was dependent on LKB1, we measured LKB1 activity (phosphorylation) in HCT116, SW480, and LOVO cells. Berberine treatment had no apparent effect on phosphorylation of LKB1 (Supplementary Figure 4A). We also determined that berberine had no apparent effect on phosphorylation of calcium/calmodulin-dependent protein kinase (CaMKK) (data not shown). These findings indicate that berberine activation of AMPK in CRC cell lines is independent of LKB1 and CaMKK.

Berberine Inhibits mTOR Signaling in CRC Cells

mTOR signaling controls cell survival and regulates metabolism [31]. As AMPK activation not only reprograms metabolism, it also enforces a metabolic checkpoint on the cell growth through its effects on p53 and mTOR signaling, suggesting AMPK-activating drugs might be useful for cancer therapeutics. We explored effects of berberine on the mTOR signal pathway. Berberine inhibited mTOR activity all 3 CRC lines (Figure 3A, B and C). In HCT116, 15 $\mu\text{mol/L}$ berberine was sufficient to inhibit mTOR activation within 24 hours. Consistent with the decreased mTOR activity, phosphorylation of p70S6kinase and of 4EBP1 decreased in a dose-dependent manner (Figure 3A, B and C). Treating HCT116 and LOVO cells with 30 $\mu\text{mol/L}$ berberine from 0 to 24 hours showed that the inhibition of mTOR phosphorylation and its downstream targets lagged behind activation of AMPK with the greatest inhibition of activity seen at 24 hours after berberine exposure (Figure 3D, E). These results suggest that prolonged activation of AMPK leads to inhibition of mTOR activity.

Berberine does not Inhibit AKT and ERK Activity in CRC Cells

Misregulation of AKT and ERK signaling pathways can affect mTOR activity, cell growth and tumorigenesis. Berberine had no apparent inhibitory effect on AKT or ERK activity in all three CRC cell lines (Supplementary Figure 4B). In fact there appeared to be a slight increase in AKT and ERK phosphorylation, which maybe the results of inhibition of the mTOR feedback regulation of AKT activity [32]. Similar results were shown for AICAR, which is also an activator of AMPK [30].

Activation of AMPK by Berberine Inhibits COX-2 Activity and Increases p53 Phosphorylation *In Vitro*

COX-2 plays a causal role in colorectal cancer development in the AOM/DSS tumor induction model [33]. As previously shown by Ishikawa and Herschman [33], COX-2 expression was detected primarily in the stroma (Figure 1C). Berberine inhibited COX-2 expression in AOM/DSS treated mice (Figure 1C). Although COX-2-expression in the AOM/DSS model is mainly seen in macrophages, fibroblasts and endothelial cells, other models of CRC and in human CRC, COX-2 expression is detected in epithelial tumors [33]. To determine if COX-2 expression is dependent on AMPK activation, we treated HCT116 cells with berberine and Compound C, an AMPK inhibitor. Berberine induced AMPK activation and inhibited COX-2 expression (Figure 4A, B and C). Compound C decreased AMPK phosphorylation in untreated and berberine treated cells and reversed the inhibition of COX-2 expression in the berberine treated cells, indicating that COX-2 expression in CRC cells is regulated by AMPK.

Previous studies have shown that AMPK activation can induce phosphorylation of p53 [34,35]. We tested the ability of berberine to phosphorylate p53 by treating HCT116 colorectal cancer cells with 0, 15, 30 and 60 $\mu\text{mol/L}$ of berberine. We found that 15 $\mu\text{mol/L}$ berberine was sufficient to increase phosphorylation of p53 without increasing p53 expression (data not shown). To determine if berberine-induced p53 phosphorylation was AMPK dependent, we treated HCT116 cells with 30 $\mu\text{mol/L}$ berberine or a combination of berberine and compound C. Compound C reversed the berberine induced phosphorylation of

p53 (Figure 4A, D), suggesting that in HCT116 CRC cells, berberine induced phosphorylation of p53 is AMPK dependent.

Activation of AMPK by Berberine Inhibits oncogenic phenotypes of CRC Cells

Expression of phosphorylated AMPK is associated with increased colorectal cancer-specific survival [35] and AMPK activators, metformin and AICAR, have been shown to reduce tumor growth in mouse xenografts [36]. To determine if the oncogenetic phenotype of CRC cells can be inhibited by activation of AMPK, we measured the effects of berberine on the migration and invasion capabilities of HCT116 cells. In a migration (scratch) assay and Matrigel invasion assay, treating CRC cells with different doses of berberine had little to no effect on migration or invasion (data not shown). In anchorage independent growth assays, however, berberine inhibited colony formation of HCT116 by 90% ($P < 0.001$) compared with DMSO control group (Figure 4E and F). Inhibition of basal AMPK activity with Compound C had little effect on colony formation, suggesting the small amount of activated AMPK in untreated cells has little effect on regulating tumorigenesis. Interestingly, inhibition of berberine dependent activation of AMPK only partially restored growth in agar, suggesting that berberine inhibition of anchorage independent growth of HCT116 cells is only partially dependent on AMPK activation.

Berberine Inhibits NF- κ B Translocation and Activates Cleaved-caspase-3

We found that inhibition of anchorage independent growth by berberine treatment was only partially reversed by AMPK inhibitor Compound C, suggesting that activating AMPK is not the only mechanism for anti-cancer activity of berberine. NF- κ B activation is associated with the increased survival of cancer cells and resistance to chemotherapy, and berberine has been shown to suppress NF- κ B activity [23,37]. Figure 5 A shows that berberine inhibited phosphorylation of NF- κ B p65 in HCT116 and LOVO cells. Phosphorylation of I κ B α , which releases p65 from sequestration in the cytoplasm, and nuclear translocation of p65 were also inhibited by berberine (Figure 5B). Berberine inhibition of p65 phosphorylation was not reversed by Compound C (Figure 5C) indicating that inhibition of NF- κ B activity is independent of AMPK activation in HCT116 cells. Berberine also inhibited expression of cyclin D1 and survivin (Figure 5D), both of which are associated with increased tumorigenesis.

Berberine's anti-cancer activity in human epidermoid carcinoma is mediated through activation of caspase-3-dependent apoptosis [38]. Cleaved-caspase-3 and cleaved poly (ADP-ribose) polymerase (PARP) were activated by berberine treatment in HCT116 (Figure 5D). These data suggest that berberine has multiple anti-carcinogenic activities.

Berberine Activates AMPK and Inhibits mTOR *In Vivo*

To determine if the berberine induced activation of AMPK and inhibition of mTOR signaling seen *in vitro* can be translated to our mouse model of colon cancer, we measured AMPK and mTOR activity in AOM/DSS treated mice and quantified the immunohistochemistry slides. We found that berberine activated AMPK in both control and AOM/DSS treated mice after 14 days of berberine treatment (Day 28 after AOM) (Figure 6A, B). Interestingly, we detected little to no phosphorylation of mTOR at this time point

after AOM/DSS exposure (Figure 6A, B). By day 70, however, mTOR phosphorylation was readily detected in the epithelial tissue of the control mice treated with AOM/DSS (Figure 6A, B), suggesting that mTOR is not activated in the acute inflammatory stage shortly after DSS but is activated by chronic inflammation seen at later times after DSS exposure. More importantly, in the berberine treated mice where AMPK was strongly activated, mTOR phosphorylation was inhibited (Figure 6A, B). Berberine also induced a slight increase (not significant) in phospho-AMPK in liver, a major site for metabolism and AMPK activity (Supplementary Figure 4C), however, there was little to no effect on mTOR activity in the liver (data not shown). These findings suggest that berberine activates AMPK at an earlier time and continues to regulate AMPK activity and inhibit mTOR at later times in colon tissue of AOM/DSS induced colorectal carcinogenesis.

Berberine Inhibits NF- κ B Activity and Induces Caspase-3 cleavage *In Vivo*

In CRC cells, berberine inhibited NF- κ B p65 phosphorylation and activated caspase-3 cleavage (Figure 5A, D). In AOM/DSS treated mice, phosphorylation of p65 was detected as early as day 28 in both PBS and berberine treated mice (Figure 6C). At later times, when activation of NF- κ B remained high in the PBS treated mice, berberine treatment produced a decrease in phosphorylation of p65 (Figure 6C). Finally, berberine induced cleavage of caspase-3 in AOM/DSS treated mice (Figure 6C). These findings indicate that berberine inhibits colitis-associated colorectal carcinogenesis through multiple mechanisms.

Discussion

Berberine, one of the main alkaloids of *Rhizoma coptidis*, offers a rich source of potential chemopreventive and therapeutic properties [39,40]. In a mouse model of colorectal cancer, we evaluated the efficacy of berberine for preventing colorectal cancer. In this model, berberine reduced leucocytes infiltration, epithelial proliferation and tumor burden in mice treated with AOM/DSS. COX-2 expression and activation of mTOR and NF- κ B, induced by AOM/DSS, was also inhibited by berberine *in vivo* (Figure 7). Furthermore, berberine induced activation of AMPK both *in vivo* and *in vitro*. In CRC cells, berberine inhibition of mTOR activity, COX-2 expression and activation of p53 phosphorylation were found to be dependent on AMPK activation. Berberine did not reduce ERK or AKT activity and activation of AMPK by berberine was independent of LKB1 in CRC cell lines. Berberine also activated cleavage of caspase-3 *in vitro* and in colon tissue from AOM/DSS treated mice. These results indicate that the anti-inflammation and anti-cancer activities of berberine are, at least in part, due to activation of AMPK, which inhibits mTOR activity, COX-2 expression and activates phosphorylation of p53. The anti-tumor and chemopreventive effects of berberine appear to be mediated by activating AMPK and inhibiting NF- κ B.

AMPK Functions as a Tumor Suppressor in Colorectal Carcinogenesis

AMPK is an energy sensor, which can be activated by many factors, such as low glucose levels, fasting, and exercise [41-43]. In various cancer cells a loss of AMPK signaling has been demonstrated and AMPK activation has been proposed as a therapeutic approach to reduce cancer cell growth [4]. Activation of AMPK in response to energy deficiencies leads to the inhibition of pathways of energy utilization and stimulation of pathways of energy

production [44]. AMPK also regulates inflammation and activation of AMPK can reduce inflammation [34]. AMPK activation by anti-inflammatory drugs can suppress tumor carcinogenesis and inflammation [42]. Activation of AMPK has been linked to increased CRC survival [13], while the loss of AMPK activation in cancer cells has been demonstrated in numerous studies [4]. Berberine has beneficial effects in the treatment of diabetes and obesity, and these effects have been linked to AMPK activity [25]. LKB1 is a tumor suppressor and a major mediator of the cellular response to energy stress [41]. LKB1 activation of AMPK regulates a wide range of cellular functions that include metabolism, proliferation and cell shape [45]. Berberine activated AMPK and inhibited colon carcinogenesis in AOM/DSS induced CRC by an LKB independent mechanism.

AMPK Functions as a Tumor Suppressor by Negatively Regulating mTOR

Aberrant mTOR signaling has been long implicated in CRC [46]. mTOR signaling regulates cell growth and proliferation by regulating the energy status of the cell [45]. AMPK negatively regulates mTOR via TSC1/2-dependent and independent mechanisms, and by regulating energy levels, which serves as a checkpoint to allow cellular proliferation only when energy levels are sufficient [47]. Inactivation of mTOR has previously been demonstrated to be important for the ability of AMPK to enforce a metabolic checkpoint [48,49]. NSAIDs and known AMPK activators such as metformin, have been shown to inhibit mTOR and decreased CRC growth *in vitro* and in CRC animal models [41]. We showed that, similar to metformin, berberine inhibited mTOR activation both *in vitro* and *in vivo*. Interestingly, we found that while AMPK as activated at early times, little or no mTOR activity was detected at this time. Activated mTOR was detected at later times in AOM/DSS treated mice. These data suggest that AMPK will inhibit mTOR activation and prevent progression of progression of colitis associated cancer.

Berberine has Anti-inflammatory and Anti-cancer Properties

This study provides further evidence for the efficacy of berberine in preventing CRC. Berberine is a potent anti-inflammatory agent and may prevent colorectal cancer by modulating inflammation. Previous studies have shown that berberine reduced DSS-induced colitis, preventing DSS-induced weight loss, myeloperoxidase activity and acute epithelial damage [50]. We have extended these results to show that, in addition to inhibition of inflammation, berberine inhibits tumorigenesis in AOM/DSS treated mice. We suspect the protective effect may involve targeting NF- κ B or AMPK. We show that berberine decreased NF- κ B activity at earlier and later times, suggesting that targeting NF- κ B is responsible for inflammation protection. NF- κ B is a pro-inflammatory transcription factor that is activate in colitis associated tumorigenesis [51]. Berberine suppresses I κ B α phosphorylation, NF- κ B p65 phosphorylation and nuclear localization, and to inhibit expression of NF- κ B target genes in lung adenocarcinoma, leukemia and multiple myeloma cell lines [23]. These authors concluded that the effects of berberine may be mediated in part through the suppression of the NF- κ B activation. We showed that berberine inhibited NF- κ B activity in colitis associated CRC *in vivo* and in CRC cells *in vitro* and that inhibition of NF- κ B in CRC cells was independent of AMPK activity.

In addition to its anti-inflammation activity, berberine has anti-cancer properties. Previous study shown that berberine suppressed colon tumor growth in the HT-29 cell xenograft mouse model through inhibiting epidermal growth factor receptor activity and proliferation [16]. In human epidermoid carcinoma berberine induced caspase-3-dependent apoptosis [38]. We used the AOM/DSS induced colorectal carcinoma mouse model to evaluate the effects of berberine on tumor promotion and progression of CRC. We found that berberine decreased tumor numbers and suppressed tumor growth by decreasing proliferation (Figure 1) and increasing apoptosis (Figure 6C).

Summary

Activation of AMPK plays key role on cancer treatment and cancer prevention. AMPK can be activated by many different factors, including phosphorylation by LKB1 or calcium/calmodulin-dependent protein kinase (CaMKK), by a high AMP/ATP ratios or low glucose levels, and by small molecules or natural products. While AMPK is activated by hormones, cytokines, pharmacological agents and natural products, the mechanisms by which these agents activate AMPK are not fully understood [52]. It was reported that berberine, like metformin, activates AMPK by inhibition the respiratory chain complex 1 in the mitochondria, decreasing ATP level [53,54]. While we detected a small but not significant decrease in ATP with berberine (data not shown), the mechanism for berberine induce activation of AMPK in CRC is unclear.

Berberine inhibits colon carcinogenesis by AMPK dependent and AMPK independent mechanisms (Figure 7). The AMPK dependent mechanisms includes inhibition of mTOR signaling and inducing phosphorylation of p53, while the AMPK independent mechanisms include the inhibition of NF- κ B activity, and its downstream targets cyclin D1 and survivin. Thus, berberine has multiple targets and may serve as an alternative therapy to prevent and/or treat colorectal cancer, alone or in combination with conventional therapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AMPK	AMP-activated protein kinase
AOM	azoxymethane
DSS	dextran sulfate sodium

CRC	Colorectal cancer
UC	Ulcerative colitis
CD	Crohn's Disease
mTOR	mammalian target of rapamycin
4EBP1	eukaryotic translation initiation factor 4E binding protein 1
ACC	acetyl-CoA carboxylase
LKB1	Liver Kinase B1
COX-2	Cyclooxygenase 2
NF-κB	nuclear factor kappaB

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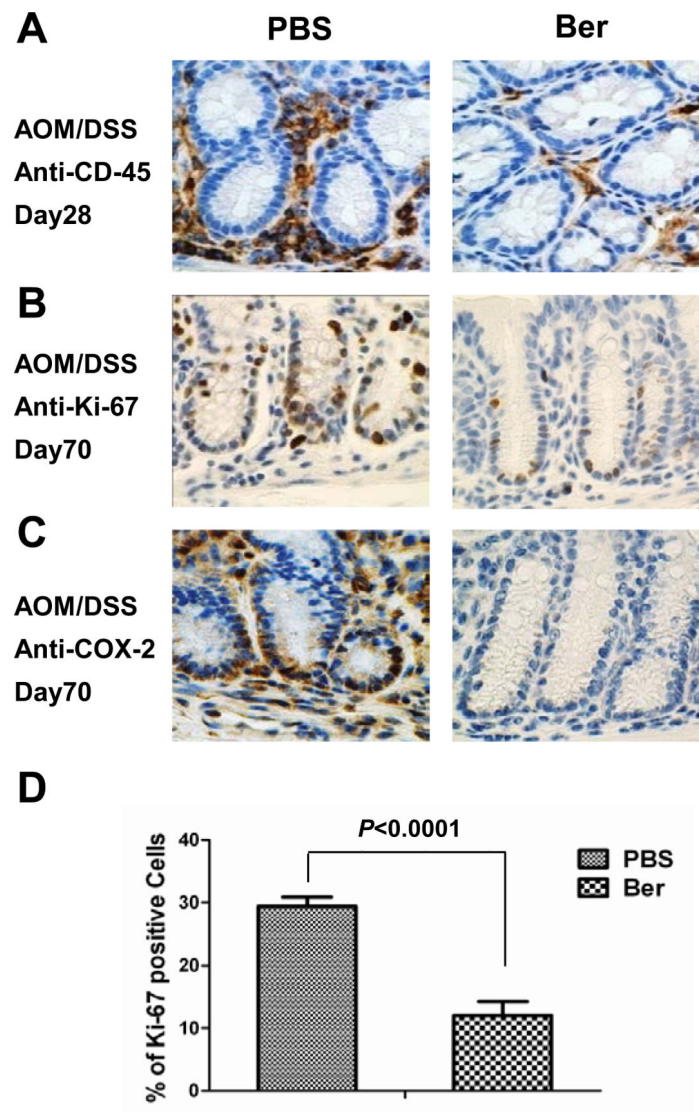


Figure 1. Berberine treatment decreases colitis associated inflammation and aberrant proliferation

Immunohistochemistry shows berberine reduced (A) CD-45⁺ leucocyte infiltration, at day 28 and (B) Ki-67⁺ aberrant proliferation and (C) COX-2 expression at day 70 post-AOM/DSS. Photomicrographs depict a representative section of colon with 5 mice in each group used for analysis. (D) Ki-67 expression quantification from immune-stain at day 70. *P* value <0.05 was considered statistically significant.

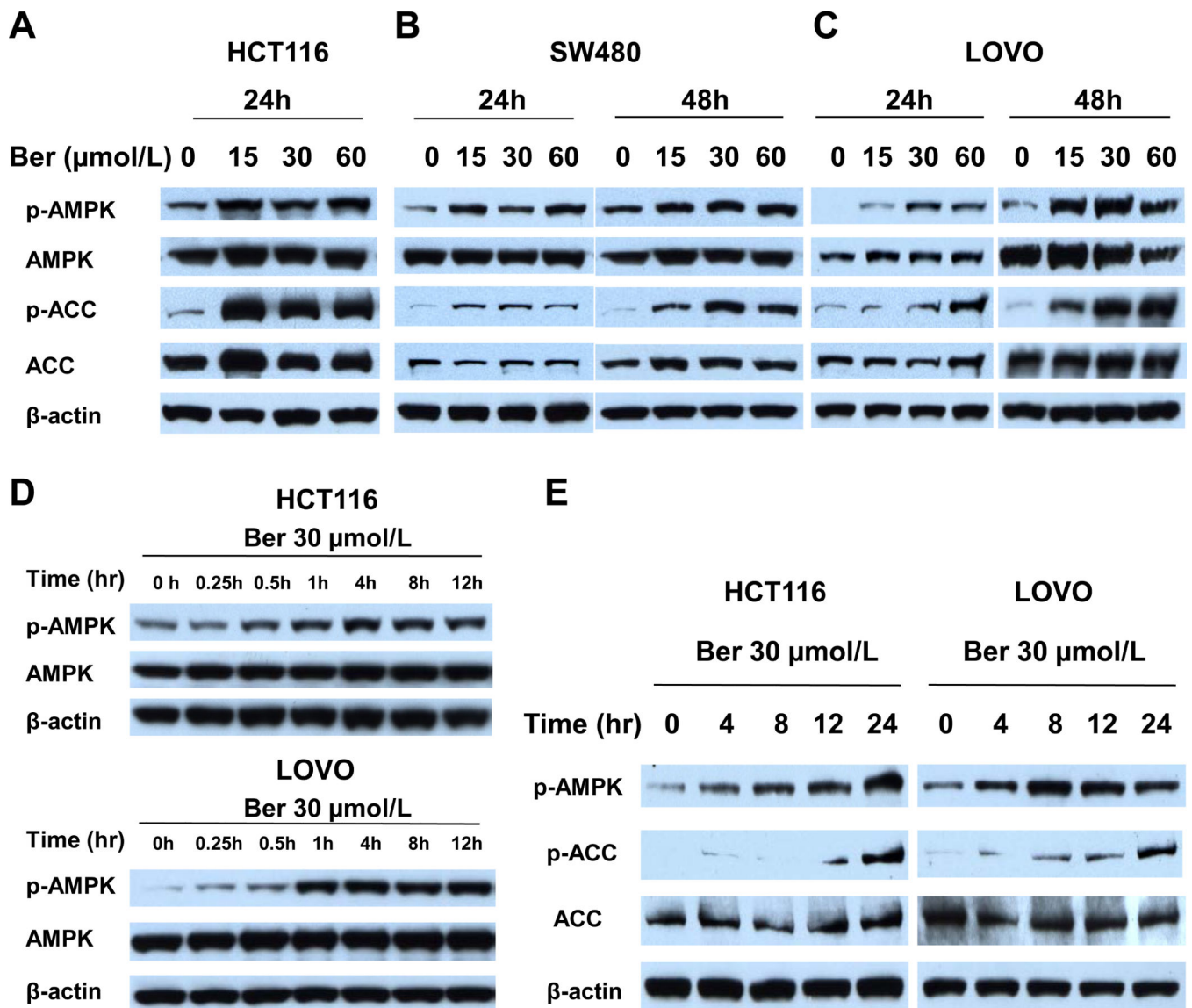


Figure 2. Berberine activates AMPK in three CRC cell lines
HCT116, SW480 and LOVO cells were seeded as 2×10^4 in 100 mm plates and after 24 hours were treated with 0.1% DMSO, 15, 30, or 60 $\mu\text{mol/L}$ berberine. After 24 and 48 hours of treatment, extracts were analyzed by Western blotting analysis. (**A-C**) Total and phosphorylated AMPK and ACC. Representative blots of three colon cancer cell lines HCT116 (**A**) SW480 (**B**) and LOVO cell (**C**). Blots are representative of three independent experiments. (**D, E**) Activation of AMPK by berberine begins as early as 1 hour post berberine treatment. Time course activation of AMPK (**D**) and ACC (**E**) in HCT116 and LOVO cells treated with 30 $\mu\text{mol/L}$ berberine from 0 hour to 24 hours and analyzed by Western blot. Phospho-AMPK (p-AMPK) and phospho-ACC (p-ACC). Blots are representative of three independent experiments.

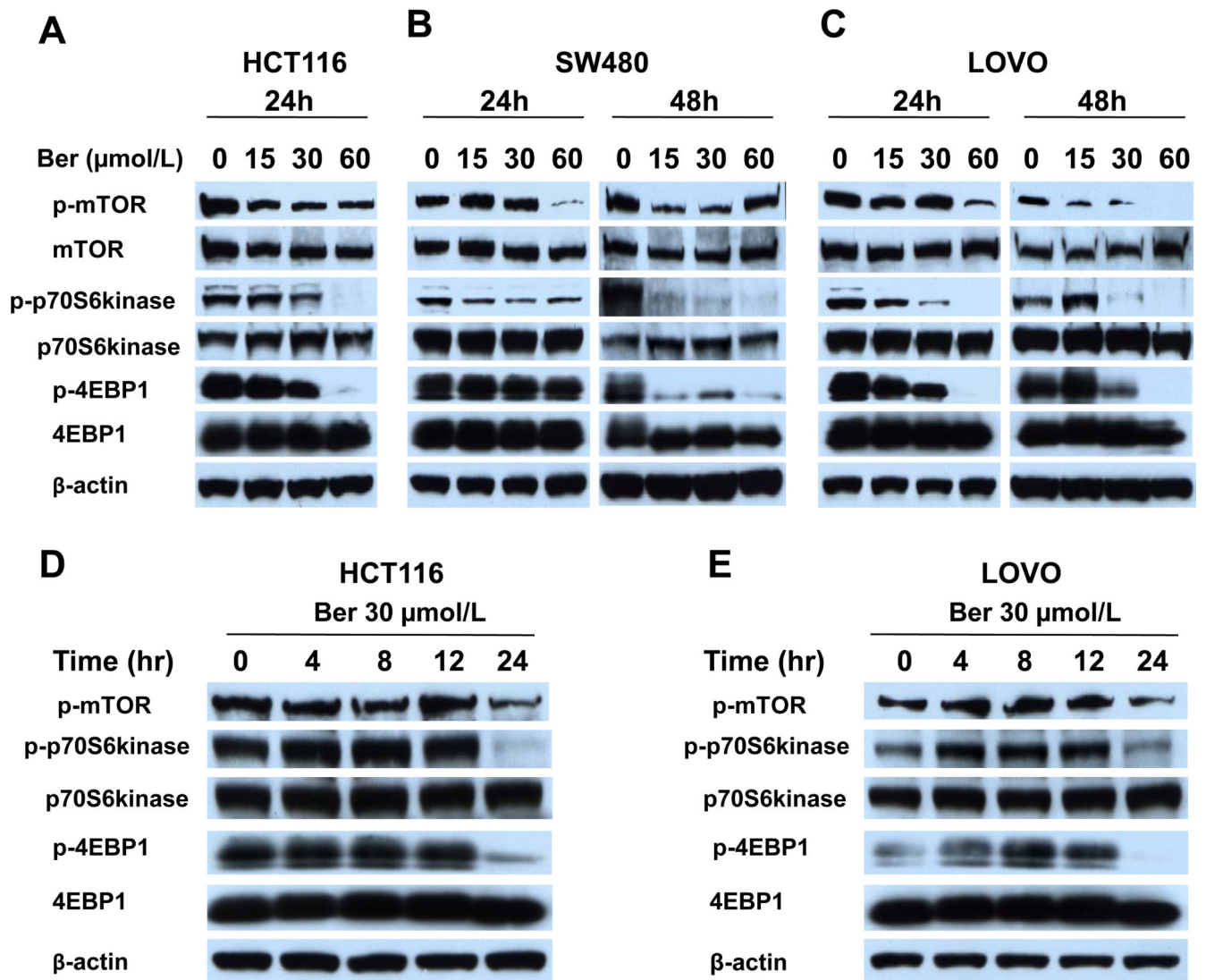


Figure 3. Berberine inhibits mTOR activity for three CRC cell lines
 HCT1116 (A) SW480 (B) LOVO (C) cells were treated with 0.1 % DMSO, 15, 30 or 60 μmol/L berberine for 24 and 48 hours. (D, E) HCT116 and LOVO cells were treated with 30 μmol/L berberine for 0 to 24 hours. Cells were lysed and analyzed by Western blotting for total and phosphorylated mTOR, p70S6kinase and 4EBP1. Representative blots from three independently performed experiments are shown. Phospho-mTOR (pmTOR), phospho-p70S6kinase (p-p70S6kinase) and phospho-4EBP1 (p-4EBP1).

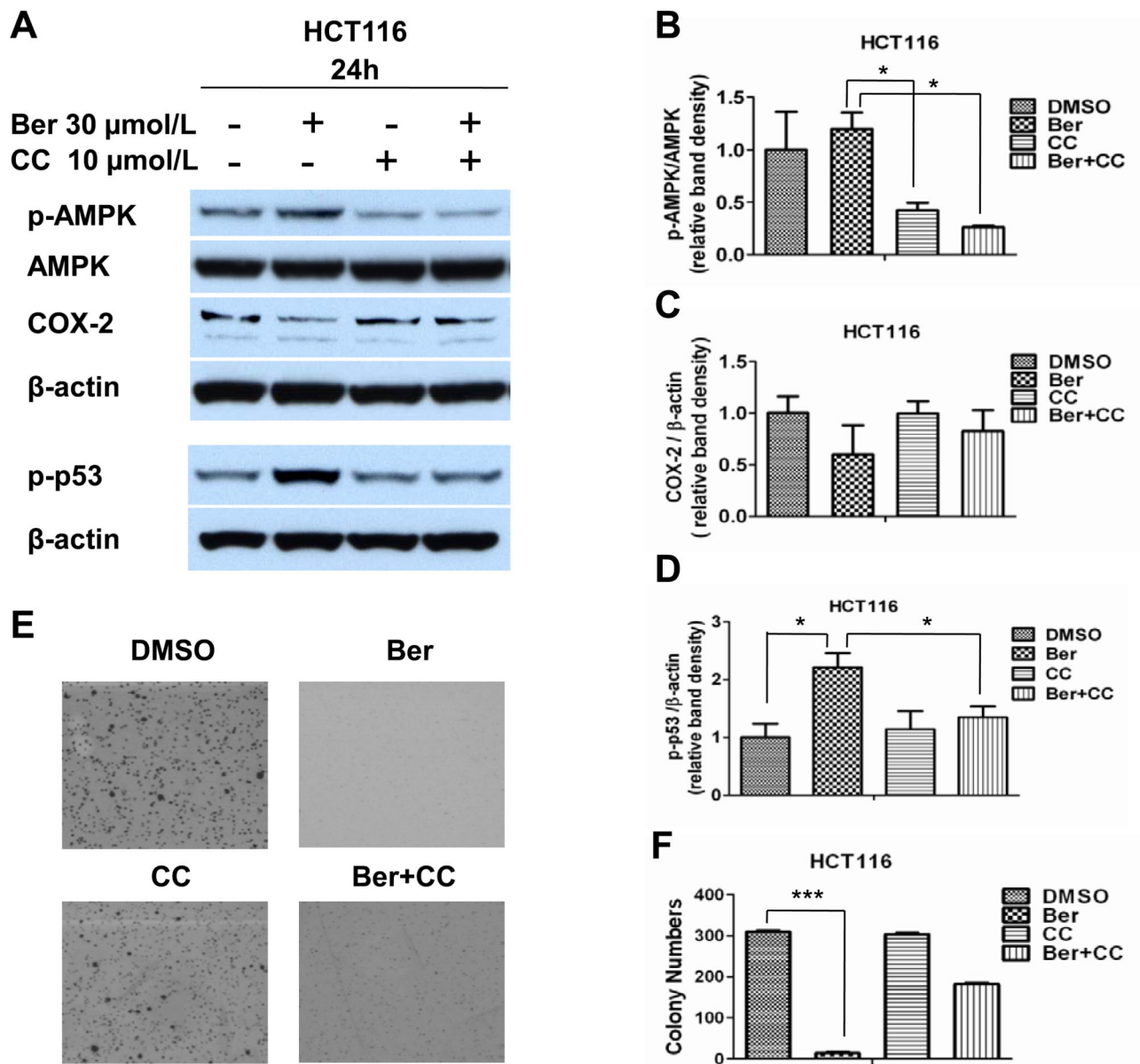


Figure 4. Berberine inhibition of COX-2 expression and anchorage independent growth, and induction of p53 phosphorylation are dependent on AMPK activation
 (A-C) HCT116 cells were treated with vehicle, berberine, Compound C or berberine plus Compound C for 24 hours. Cells were lysed and analyzed for total and phosphorylated AMPK and p53, and for COX-2 expression (A) Densitometry analyses of Western blots were determined by Image J to quantify the relative protein levels of (B) p-AMPK versus AMPK and (C) COX-2 and (D) p-p53 versus β -actin. Three independent experiments were analysis. $*p < 0.05$ versus berberine treated cells. (E, F) HCT116 cells were treated with vehicle, berberine, Compound C, or berberine plus Compound C for 10 days in soft agar and the average colony numbers were counted. Column, mean of triplicate samples, bars, standard deviation, $***p < 0.001$ versus DMSO control cells. Vehicle (DMSO 0.1%), berberine (Ber), Compound C (CC) and combination berberine and Compound C (Ber+CC).

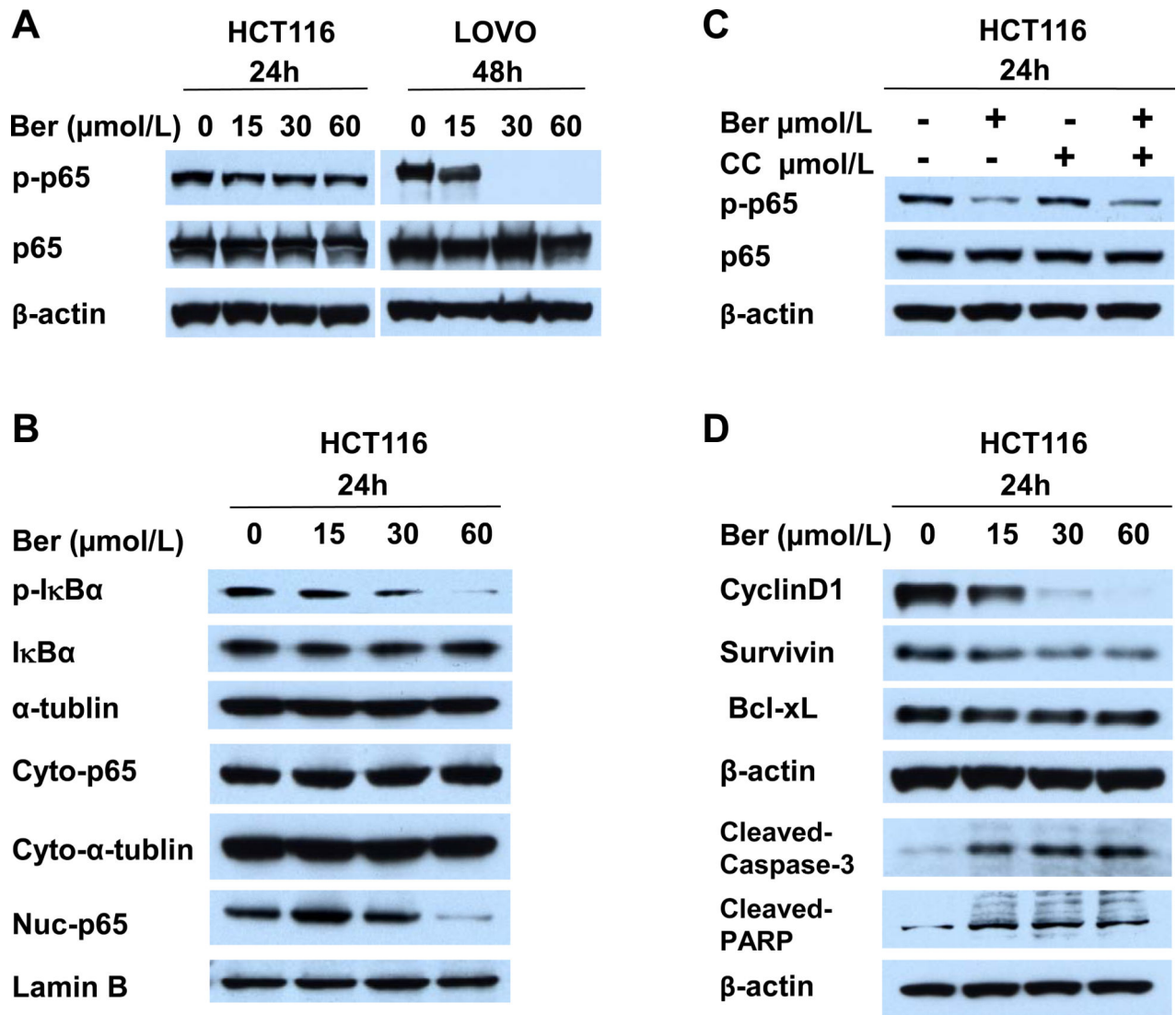


Figure 5. Berberine inhibits NF-κB activity and activates cleaved-caspase-3
(A-B) Berberine inhibits phosphorylation of p65. HCT116 and LOVO cells were treated with 0 to 60 μmol/L berberine for 24 and 48 hours. **(A)** Total p65 and phosphorylated p65 (pp65) analyzed by Western blot. **(B)** Berberine inhibits phosphorylation of IκBα and nuclear localization of p65. HCT116 cells were treated with 0 to 60 μmol/L berberine for 24 hours and the cytoplasmic extracts were analyzed for total and phosphorylated IκBα levels. Cytoplasmic and nuclear extracts were analyzed for cytoplasmic (Cyto-p65) and nuclear (Nuc-p65) localization of p65. α-tubulin and Laminin B were used as cytoplasmic and nuclear markers respectively. Blots are representatives of three independent experiments. **(C)** Activation of NF-κB is AMPK independent. HCT116 cells were treated with DMSO, berberine, Compound C, and berberine plus Compound C for 24 hours. Total and phosphorylated p65 was analyzed Western blot. Blots are representatives of three independent experiments. **(D)** Expression of candidate NF-κB targets Cyclin D1 and Survivin were inhibited by berberine. HCT116 cells were treated with 0 to 60 μmol/L berberine for 24 hours. Cyclin D1, Survivin and Bcl-xL expression was analyzed by

Western blot. Berberine activated cleavage of caspase-3 and PARP. HCT116 cells were treated with 0 to 60 $\mu\text{mol/L}$ berberine for 24 hours. Cleaved caspase-3 and PARP were analyzed by Western blot. Blots are representatives of three independent experiments.

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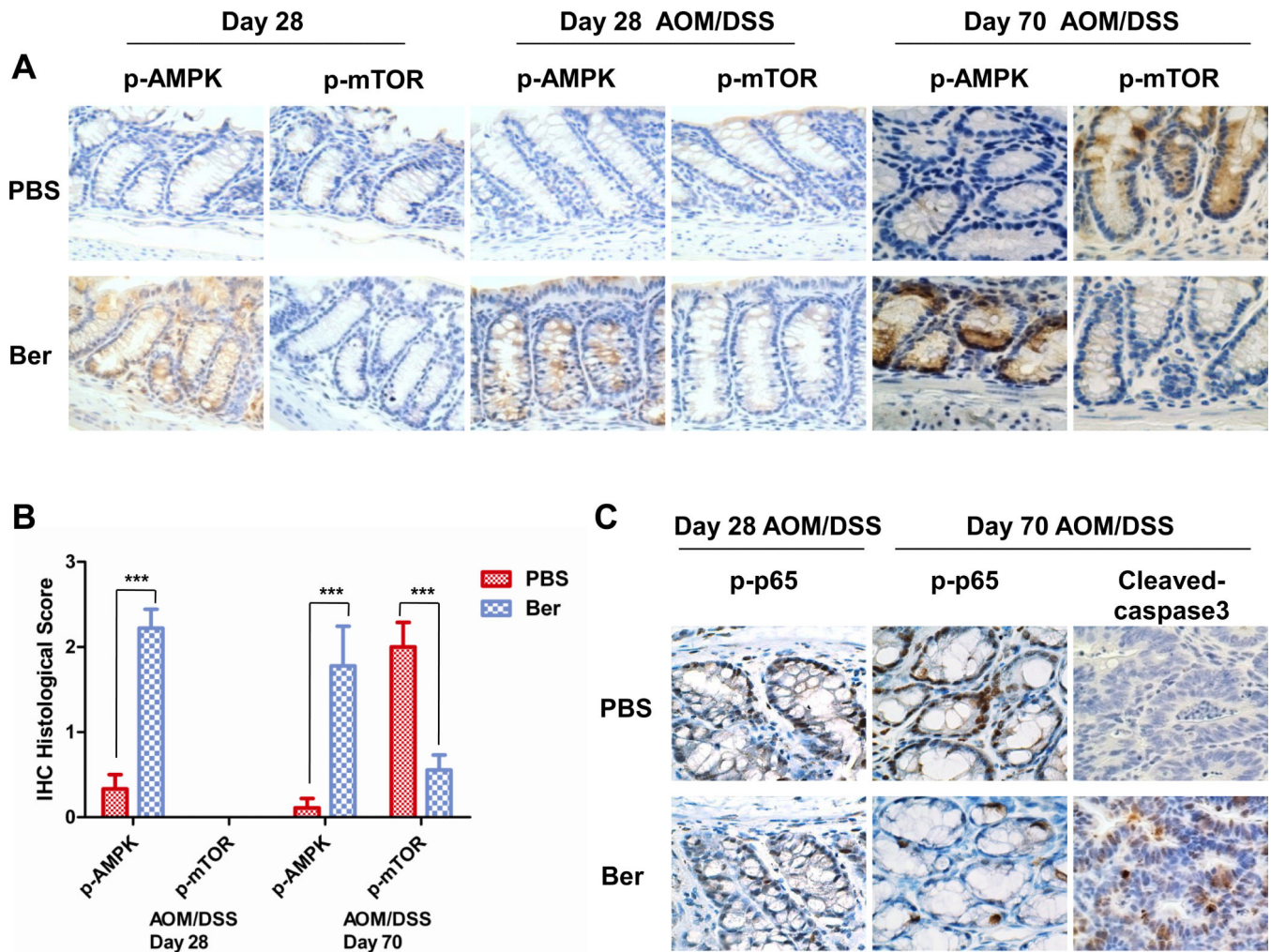


Figure 6. Berberine activates AMPK signal pathway and inhibits NF- κ B *in vivo*
(A) AMPK was activated by berberine in AOM/DSS untreated and treated mice. Immunohistochemistry staining with phospho-AMPK (p-AMPK), phospho-mTOR (p-mTOR) of colon tissue on Day 28 showed berberine induced activation of AMPK. Day 70 showed sustained activation of AMPK and inhibition of AOM/DSS induced mTOR activity in berberine treated mice. Representative pictures (60 \times) of each group are shown. Colon tissues from 5 mice in each group were analyzed. **(B)** Immunohistochemical phenotypes were assessed by a semi-quantitative scoring method classified into four categories: negative=0, poorly positive=1, positive =2 and strongly positive=3 according to the intensity of staining and specificity. Data are shown as the mean score for 5 mice with standard deviation, *** $p < 0.001$ versus PBS treatment group. **(C)** Immunohistochemistry staining with phospho-p65 (p-p65) showed inhibition of p-p65 by berberine 70 days after AOM/DSS treatment. Berberine induced cleavage of caspase-3 was also detected by immunohistochemistry at day 70. Representative pictures (60 \times) are shown. Colon tissues from 5 mice in each group were used for analysis.

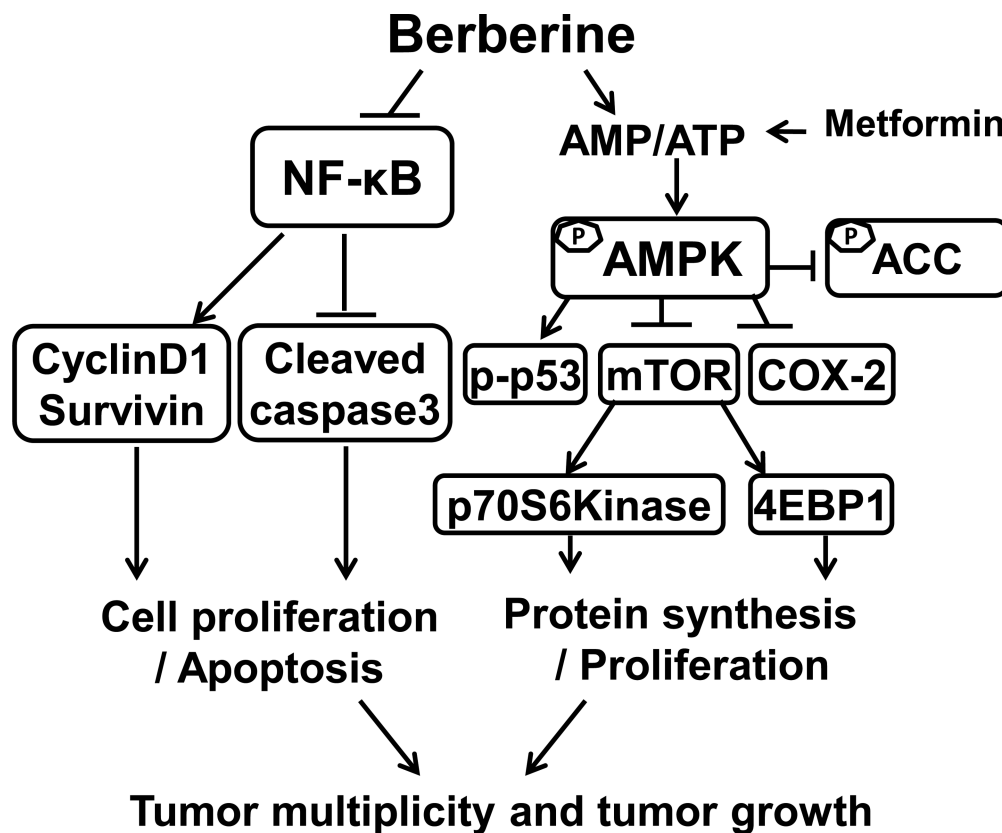


Figure 7. Mechanisms of berberine in suppressing colon cancer

Berberine activated AMPK negatively regulates the mTOR signal pathway, resulting in inhibition of colon cancer proliferation and growth. Activated AMPK negatively regulates COX-2 in colon cancer. Activation of AMPK also induces phosphorylation of p53 which is a tumor suppressor. Inhibition of tumorigenesis by berberine is partially dependent on inhibition of the NF-κB signaling pathway. This NF-κB suppression results in inhibiting transcription of NF-κB targets CyclinD1 and Survivin and stimulating the cleavage of caspase-3, modulating cell proliferation and apoptosis. Interactions leading to activation of molecular targets are indicated by arrows; those that inhibited are indicated by a bar. Berberine modulation of both pathways leads to inhibition of tumor multiplicity and tumor growth.

Table 1Effect of berberine on tumor multiplicity (Mean \pm SEM)

	Mice N	Tumor Number N/mouse	Tumor size [†]			Colon Length (cm)
			<2mm	2-4mm	>4mm	
Control (PBS)	10	13.6 \pm 2.4	9.6 \pm 1.7	3.3 \pm 0.7	3.3 \pm 0.7	5.4 \pm 0.2
Berberine	10	5.4 \pm 1.3	5.0 \pm 1.1	0.2 \pm 0.2	0.0 \pm 0.0	6.3 \pm 0.4
<i>P</i> Group Differences*		0.0009	0.05	0.001	0.02	0.03

FVB mice were treated with 40mg/kg berberine or PBS vehicle for 10 weeks, then collected the colon tissue and observed tumor numbers and measured tumor size under microscopy. Data was presented are mean \pm SEM.

* *P*-values are compared to PBS vehicle.

[†] Average tumor numbers per mouse classified by tumor size. 95% Confidence Interval for different tumor size range: tumor size <2mm, 2.46-13.46; tumor size 2-4mm, -0.25-4.99; tumor size >4mm, 0.00-1.29.