Berberine Regulates AMP-Activated Protein Kinase Signaling Pathways and Inhibits Colon Tumorigenesis in Mice

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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-κB) 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-κB 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-κB. © 2014 Wiley Periodicals, Inc.

Key words: 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

**Abbreviations:** AOM/DSS, azoxymethane initiated and dextran sulfate sodium; ADM, azoxymethane; DSS, dextran sulfate sodium; AMPK, AMP-activated protein kinase; mTOR, mammalian target of rapamycin; LKB1, liver kinase B1; NF-κB, nuclear factor kappaB; CRC, colorectal cancer; UC, ulcerative colitis; CD, Crohn’s disease; 4EBP1, eukaryotic translation initiation factor 4E binding protein 1; H&E, hematoxylin-eosin; ACC, acetyl-CoA carboxylase; COX-2, Cyclooxygenase 2.

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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-inflammatory, 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-κB) 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 S1), 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). Fetal Bovine Serum (FBS) was obtained from Atlantic Biological. TACS MTT Cell Proliferation Assay kits were obtained from Trevigen (Helgerman, Gaithersburg, MD). Azoxymethane was obtained from Sigma–Aldrich (St. Louis, MO). Dextran Sodium Sulfate, 36000 to 50000 kDa, was obtained from MP Biomedicals LLC, (Solon, OH). NE-PER Nuclear and Cytoplasmic Extraction Kit was ordered from Thermo Scientific (Rockford, IL). Annexin V-FITC Apoptosis Detection Kit was ordered from BD Biosciences (San Diego, CA).

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-κB 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); anti-β-actin was from Sigma–Aldrich; anti-Ki-67 and anti-CD-45 were from Abcam (San Francisco, CA).

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-wk-old) were purchased from Charles River (Frederick, MD). Animal Diets were from Teklad Lab. After 1 wk accustomed to the new environment and controlled diet (AIN93G), all the mice were randomly divided into four 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 S2), 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 d. Berberine treatment began 1 d 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, five times a week for two continuous weeks (Days 15–19 and 23–27), followed by three times a week (Days 30–70) until to the end of the study. On Day 28 after AOM injection, five 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 wk 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. Liver samples were collected and the size were determined. Colon tissue was evaluated by 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-μm sections, and put onto microscopic slides by Histoserv, Inc. (Germantown, MD). 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-μm thick paraffin embedded sections were obtained from Histoserv, Inc. 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 min and incubated in normal goat serum for 1 h. Sections were incubated overnight in primary antibody solution diluted as follows: Ki-67, CD45, 1:100; p-mTOR, p-AMPK, and COX-2, 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, Burlingame, CA), detected with Diaminobenzidine (Sigma) 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, Bethesda, MD). Representative images were captured from five independently samples.

Cell Culture

HCT116, SW480, and LOVO colon cancer cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and Division of Cancer Treatment and Diagnosis (DCTD) Tumor Repository (National Cancer Institute). 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 × 10⁴ cells per well in a flat-bottomed 96-well plate and cultured for 24 h. Cells were treated with berberine (0–60 μmol/L) or DMSO (0.1%) for 24 or 48 h. The media was removed and cells were washed with cold PBS on ice. Whole-cell extracts were prepared in RIPA lysis buffer (Thermo Scientific) supplemented with proteinase inhibitors (Roche, Mannheim, Germany). Protein concentration was determined by Micro BCA Protein Assay Kit (Thermo Scientific). Thirty microgram of total protein was denatured, heated for 5 min, and fractionated on a 10% SDS-PAGE. Proteins were transferred to Nitrocellulose membrane (Bio-Rad, Hercules, CA) for 2 h at 300 mA, blocked and incubated with a primary specific antibody in 5% non-fat milk for 1 h 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 h. Antigens were detected with Super Signal West Femto Maximum Sensitivity Substrate (Thermo Scientific) 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 × RPMI media. Cells were treated with DMSO (0.1%) vehicle, berberine (30 μmol/L), Compound C (10 μmol/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 d after which the colonies were stained with Nitroblue tetrazolium Blue chloride (NBT), scanned and counted with GelCount (Oxford Optronix Ltd,
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 expression of CD-45, 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 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 μmol/L) for 24, 48, and 72 h. Berberine treatment caused a concentration and time-dependent inhibition of cell growth (Figure 2A). Flow cytometric analysis revealed a significant increase in sub-G1 population in berberine treated groups compared to the PBS treated groups. The results indicated that berberine induced apoptosis by cell cycle arrest in the G0/G1 phase (Figure 2B). The expression of pro-apoptotic proteins, such as Bax and Bcl-2, were increased with the increase in berberine concentration (Figure 2C). This suggests that berberine inhibited AOM/DSS-induced proliferation and apoptosis, preventing tumorigenesis.
These results suggest that berberine has strong effects 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 in the number of apoptotic HCT116 and SW480 cells (Supplementary Figure S3D and 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 μmol/L) on AMPK activation in 3 CRC cell lines. Berberine increased phosphorylation of AMPK at Thr172, which reflects AMPK activation, at 24 h in HCT116 and at 24 and 48 h in SW480 and LOVO cells (Figure 2A–C). HCT116 cells treated with berberine for 48 h showed significant loss of attachment to the dish. The 15 μmol/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–C).

In a time-dependence experiment HCT116 and LOVO cells were treated with 30 μmol/L berberine from 0 to 24 h. AMPK phosphorylation was detected as early as 30 min after berberine treatment (Figure 2D and E) and continued to increase up to 24 h. 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 S4A). 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]. AMPK activation not only reprograms metabolism but also enforces a metabolic checkpoint on 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–C). In HCT116, 15 μmol/L berberine was sufficient to inhibit mTOR activation within 24 h. Consistent with the decreased mTOR activity, phosphorylation of p70S6 kinase and of 4EBP1 decreased in a dose-dependent manner (Figure 3A–C). Treating HCT116 and LOVO cells with 30 μmol/L berberine from 0 to 24 h 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 h after berberine exposure (Figure 3D and 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 S4B). In fact there appeared to be a slight increase in AKT and ERK phosphorylation which may be the result 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, in other models of CRC and in human CRC, COX-2 expression is detected in epithelial cells and 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–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 μmol/L of berberine. We found that 15 μmol/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 μmol/L berberine or a combination of berberine and compound C. Compound C reversed the berberine induced phosphorylation of p53 (Figure 4A and 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 tumor phenotypes 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 or no effect on migration or invasion or berberine and compound C. Compound C reversed the berberine induced phosphorylation of p53 (Figure 4A and D), suggesting that in HCT116 CRC cells, berberine induced phosphorylation of p53 is AMPK dependent.
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 partially dependent on AMPK activation.

Berberine Inhibits NF-κB 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-κB activation is associated with the increased survival of cancer cells and resistance to chemotherapy, and berberine has been shown to suppress NF-κB activity \([23,37]\).

Figure 5 A shows that berberine inhibited phosphorylation of NF-κB p65 in HCT116 and LOVO cells. Phosphorylation of IκBα, which releases p65 from

Molecular Carcinogenesis

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**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 24h. 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. (EF) HCT116 cells were treated with vehicle, berberine, Compound C, or berberine plus Compound C for 10 d 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).
sequestration in the cytoplasm, and nuclear translo-
cation of p65 were also inhibited by berberine (Figure 5B). Berberine inhibition of p65 phosphoryla-
tion was not reversed by Compound C (Figure 5C) indicating that inhibition of NF-κB activity is inde-
pendent 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 epider-
moid 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
mice and quantified the IHC slides. We found
that berberine activated AMPK in both control and
AOM/DSS treated mice after 14 d of berberine
treatment (Day 28 after AOM) (Figure 6A and B).
Interestingly, we detected little or no phosphoryla-
tion of mTOR at this time point after AOM/DSS
exposure (Figure 6A and B). By Day 70, however, mTOR phosphorylation was readily detected in the epithelial tissue of the control mice treated with AOM/DSS (Figure 6A and 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 and B). Berberine also induced a slight increase (not significant) in phospho-AMPK in liver, a major site for metabolism and AMPK activity (Supplementary Figure S4C); however, there was little or 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-κB Activity and Induces Caspase-3 Cleavage In Vivo

In CRC cells, berberine inhibited NF-κB p65 phosphorylation and activated caspase-3 cleavage (Figure 5A and 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-κB 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.

Figure 6. Berberine activates AMPK signal pathway and inhibits NF-κB 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×) 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 d after AOM/DSS treatment. Berberine induced cleavage of caspase-3 was also detected by immunohistochemistry at Day 70. Representative pictures (60×) are shown. Colon tissues from 5 mice in each group were used for analysis.
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 carcinogenesis, we evaluated the efficacy of berberine for preventing colorectal cancer. In this model, berberine reduced leucocyte infiltration, epithelial proliferation, and tumor burden in mice treated with AOM/DSS. COX-2 expression and activation of mTOR and NF-κB, induced by AOM/DSS, was also inhibited by berberine in vivo (See Figure 7 for pathway diagram). Furthermore, berberine induced activation of AMPK both in vivo and in vitro. In CRC cells, berberine inhibition of mTOR activity and 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 and 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-κB.

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 [27]. 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.

**Figure 7.** Mechanisms of berberine in suppressing colon cancer. Berberine 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.
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 inhibiting inflammation, berberine inhibits tumorigenesis in AOM/DSS treated mice. We suspect the protective effect may involve targeting NF-κB or AMPK. We show that berberine decreased NF-κB activity at earlier and later times, suggesting that targeting NF-κB is responsible for inflammation protection. NF-κB is a pro-inflammatory transcription factor that is activated in colitis associated tumorigenesis [51]. Berberine suppresses IkBα phosphorylation, NF-κB p65 phosphorylation, and nuclear localization and inhibits expression of NF-κB 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-κB activation. We showed that berberine inhibited NF-κB activity in colitis associated CRC in vivo and in CRC cells in vitro and that inhibition of NF-κB in CRC cells was independent of AMPK activity.

In addition to its anti-Inflammation activity, berberine has anti-cancer properties. Previous studies showed 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 a key role in 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 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]. Berberine, like metformin, activates AMPK by inhibiting 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 induced activation of AMPK in CRC is unclear.

Berberine inhibits colon carcinogenesis by AMPK dependent and AMPK independent mechanisms (Figure 7). The AMPK dependent mechanisms include inhibiting mTOR signaling and inducing phosphorylation of p38, while the AMPK independent mechanisms include inhibiting NF-κB 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.

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REFERENCES


19. Li ET AL. Molecular Carcinogenesis


25. Li ET AL. Molecular Carcinogenesis


30. Li ET AL. Molecular Carcinogenesis

31. Liu ET AL. Molecular Carcinogenesis


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