

Fluorinated N,N'-Diarylureas As Novel Therapeutic Agents Against Cancer Stem Cells

Dasha E. Kenlan¹, Piotr Rychahou^{1,2}, Vitaliy M. Sviripa³, Heidi L. Weiss¹, Chunming Liu³, David S. Watt³, and B. Mark Evers^{1,2}



Abstract

Colorectal cancer is the second-leading cause of cancer-related mortality in the United States. More than 50% of patients with colorectal cancer will develop local recurrence or distant organ metastasis. Cancer stem cells play a major role in the survival and metastasis of cancer cells. In this study, we examined the effects of novel AMP-activated protein kinase (AMPK) activating compounds on colorectal cancer metastatic and stem cell lines as potential candidates for chemotherapy. We found that activation

of AMPK by all fluorinated N,N'-diarylureas (FND) compounds at micromolar levels significantly inhibited the cell-cycle progression and subsequent cellular proliferation. In addition, we demonstrated that select FNDs significantly increased apoptosis in colorectal cancer metastatic and cancer stem cells. Therefore, FNDs hold considerable promise in the treatment of metastatic colorectal cancer, through elimination of both regular cancer cells and cancer stem cells. *Mol Cancer Ther*; 16(5); 831–7. ©2017 AACR.

Introduction

Colorectal cancer is the second leading cause of cancer deaths in the United States (1). This high mortality rate is largely attributed to tumor metastasis and recurrence (2). At initial presentation, more than half of all patients display metastatic disease in regional lymph nodes or systemic organs (e.g., liver). The five-year survival rate for patients with metastatic colorectal cancer is approximately 13%, as compared to 90% for nonmetastatic disease (1). A growing body of evidence suggests that cancer stem cells play a role in the resistance and spread of cancer cells (2–4). Cancer stem cells are a subpopulation of tumor cells that are capable of self-renewal and display resistance to DNA damage-induced cell death, a ruinous property that allows metastatic tumors to survive initial treatments and repopulate. Developing new antineoplastic agents that selectively target cancer stem cells represents a potentially valuable treatment to improve colorectal cancer outcomes (2–4).

Cancer stem cells possess demanding metabolic requirements and survival mechanisms that are potential targets for treatment (5). One such critical pathway involves AMP-activated protein kinase (AMPK), which coordinates growth, autophagy, and metabolism (6). AMPK acts as a metabolic sensor for the ratio of AMP to ATP in cells and has several downstream transcriptional targets (6). Among the antineoplastic effects of AMPK activation are cell-cycle arrest through stabilization of p53 and cyclin-dependent kinase inhibitors p21^{waf1} and p27^{kip1}, inhi-

tion of macromolecular synthesis, inhibition of mTORC1, and inhibition of the Warburg effect (7, 8). In support of the particular importance of AMPK in cancer cells and cancer stem cells, the widely studied AMPK activator, metformin, reduces cancer incidence in diabetic populations for many different cancers, including colorectal cancer (9). Furthermore, *in vitro* and *in vivo* studies with metformin specifically show cytotoxicity toward cancer stem cells (10, 11).

We identified the antineoplastic effects of fluorinated N,N'-diarylureas (FND) in a high-throughput screening program (12) and found that several potent FNDs inhibited growth of colorectal cancer cell lines through inhibition of the mTOR pathway (12). Overexpression of mTORC1 and mTORC2 components, Raptor and Rictor, is important to tumorigenesis (13), and the activation of AMPK regulates cell growth by suppressing mTORC1 through direct phosphorylation of the tumor suppressor, TSC2, and Raptor (6). Through this mechanism, we anticipated that AMPK activation would directly inhibit colorectal cancer cell proliferation.

In this study, we investigated the ability of eight FNDs to inhibit growth and induce apoptosis in colorectal cancer metastatic cell lines and stem cells. Activation of AMPK by all FND compounds successfully inhibited cell-cycle progression and subsequent cellular proliferation. These results demonstrate that FNDs exhibit considerable promise in the treatment of metastatic colorectal cancer, predominantly through the inhibition of colorectal cancer stem cells.

Materials and Methods

Fluorinated N,N'-diarylureas

FNDs were synthesized as previously described (12). Table 1 shows the FNDs used in this study. Stock solutions (10 mmol/L) in DMSO were stored at -20°C .

Cell lines and culture maintenance

Human cell line HT29 was purchased from ATCC. KM20 was provided by Dr. Isaiah J. Fidler (M.D. Anderson Cancer Center,

¹Lucille Parker Markey Cancer Center, University of Kentucky, Lexington, Kentucky. ²Department of Surgery, University of Kentucky, Lexington, Kentucky. ³Department of Molecular and Cellular Biochemistry, University of Kentucky, Lexington, Kentucky.

Corresponding Author: B. Mark Evers, University of Kentucky, 800 Rose Street, CC140, Lexington, KY 40536. Phone: 859-323-6556; Fax: 859-323-2074; E-mail: mark.evers@uky.edu

doi: 10.1158/1535-7163.MCT-15-0634

©2017 American Association for Cancer Research.

Houston, TX); STR validation analysis (Bio-Synthesis) yielded a full DNA profile and no contamination by another human cell line was detected; there were no matches after searching ATCC, JCRB, or DSMZ databases. HCT116 *pik3ca* wild-type and mutant cell lines were a gift from Dr. J. Wang (14). Human colorectal cancer stem cell line 1 (no. 36112-39; lot no. 12121800-05) and stem cell line 2 (no. 36112-39; lot no. 1313161-12) were purchased from Celprogen. Cancer stem cells were limited to less than 12 passages.

Cell lines were routinely grown as monolayer cell cultures in 5% CO₂ in air, and 100% relative humidity at 37°C. HT29 and KM20 cell lines were grown in McCoy's 5A medium (Sigma-Aldrich) and supplemented with 10% FBS and 1× antibiotic-antimycotic (Life Technologies). Stem cell lines were grown in Cancer Stem Cell Complete Growth Media with Serum without antibiotic on precoated flasks with Human Colon Cancer Stem Cell Extra-cellular Matrix (both from Celprogen). Cell passages were carried out by detaching adherent cells in a logarithmic growth phase by addition of a mixture of 0.25% trypsin with 0.02% EDTA (Sigma Aldrich) and incubating for 10 to 15 min at 37°C. The number of viable cells was estimated with a cell counter V-CELL XR (Beckman Coulter). Metformin HCl was purchased from Selleckchem.

Cytotoxicity SRB assay

For each experiment, cell lines were seeded in two 96-well plates in normal medium (5×10^3 cells/well, 100 μ L). At 24 hours, 100 μ L of media with drugs at different concentrations were added to each well. DMSO was used as a treatment control. Cell viability was measured using the Cytoscan-SRB Cell Cytotoxicity Assay (G-Biosciences) according to manufacturer's instructions. Cell viability was plotted as a percentage relative to DMSO treatment alone.

Western blot analysis and antibodies

Total protein lysates were resolved on a 4%–12% Bis-Tris gel and transferred to Immobilon PVDF transfer membranes. Membranes were incubated for 1 hour at room temperature in blocking solution (TRIS-buffered saline containing 10% nonfat dried milk and 0.1% Tween 20), followed by an overnight incubation in primary antibodies at 4°C. Membranes were washed three times and incubated with horseradish peroxidase-conjugated secondary antibodies for 1 hour. After three additional washes, the immune complexes on the membranes were visualized using Immobilon Western Chemiluminescent HRP substrate (EMD Millipore) or Amersham ECL (GE Life Sciences).

Antibodies for Western blot analysis included the following: PARP (#9542, 1:1,000), Phospho-AMPK α (#2531, Thr172, 1:1,000), phospho-S6 ribosomal protein (Ser235/236; Cell Signaling); cyclin D1 (Abcam; #AB34175, 1:5,000); β -actin (Sigma Aldrich, #A5441, 1:20,000); anti-rabbit and anti-mouse (Santa Cruz Biotechnology, #SC-2054, #SC-2055, 1:3,000).

Patient tumor engraftment into SCID mice and PDX cell line establishment

The original patient colorectal cancer tumor (F0 generation) was divided and implanted into the flanks of NOD scid gamma mice (The Jackson Laboratory; 005557). When the resulting tumors (F1 generation) grew to 1 cm³, they were resected, divided into 2-mm³ pieces, and implanted into 5 mice (F2

generation). All animal studies were performed in accordance with policies of the Institutional Animal Care and Use Committee and were approved by the Institutional Review Board of the University of Kentucky.

Liberase DH Research Grade (05401054001; Roche Applied Science) was resuspended in sterile water at a 2.5 mg/mL concentration and stored in single-use 100 μ L aliquots at –80°C. collagenase/hyaluronidase (07912; StemCell Technologies) was aliquoted into single-use 250 μ L aliquots and stored at –80°C. Upon collection, PDX tumors (F2 generation) were placed into DMEM complete cell culture media supplemented with 1× Gibco antibiotic-antimycotic (15240-062; Life Technologies) for transportation. Tumor fragments were minced into 2-mm cubes using scissors and digested in 50 μ g/mL Liberase DH (100 μ L) and 0.5× collagenase/hyaluronidase (250 μ L), diluted in 5 mL of DMEM serum-free media for 4 hours at 37°C with gentle agitation by magnetic stirring bar. No undigested tissue was observed; cells were washed twice with complete cell culture media and transferred into 10% FBS DMEM media supplemented with 1× Gibco antibiotic-antimycotic.

Statistical analysis

Analysis of variance was used for comparison of cell viability across DMSO and varying dose levels of FND treatment, and adjusted *P* values using the Holm's procedure were calculated for pairwise comparison of each dose level with DMSO.

Results

Treatment with FNDs induces cell-cycle inhibition and apoptosis in metastatic colorectal cancer cells

We performed a high-throughput screen of *N,N'*-diarylureas to identify potent AMPK activators and selected eight active agents (Table 1; ref. 12). Next, HT29 cells were treated with the eight agents (FND 4a, 4b, 4c, 4d, 4e, 4f, 4g, 4h) for 48 hours. Decreased cell viability was evident at a drug dose as low as 5 μ mol/L, and was below 50% for all eight compounds treated at the 25 μ mol/L dose (Table 2). To determine whether the prominent decrease in cell viability was due to cell-cycle inhibition or apoptosis induction, KM20 and HT29 colorectal cancer cells were treated with each of the eight FNDs for 24 hours at 25 or 50 μ mol/L. Cyclin D1 expression and PARP cleavage were analyzed by Western blot analysis. Cyclin D1 expression was drastically decreased in both cell lines after treatment with FND 4b, 4c, 4d, 4e, 4f, 4g, and 4h at 25 μ mol/L (Fig. 1A). Prominent PARP cleavage was observed after treatment at the 50 μ mol/L dose, notably after treatment with the 4b compound (Fig. 1B).

Treatment with FNDs induces cell-cycle inhibition and apoptosis in cancer stem cells

Colorectal cancer stem cell lines were treated with FND 4b, 4d, 4f, 4g, and 4h at 50 μ mol/L for 24 hours and analyzed for cyclin D1 expression and PARP cleavage. Cyclin D1 expression was decreased in all treatment groups; prominent PARP cleavage was observed in both stem cell lines only after treatment with FND 4b (Fig. 2A). Activation of apoptosis is especially important for elimination of cancer stem cells, because these cells are progenitors for tumor progression; FND 4b treatment induced apoptosis in both metastatic cancer cells and cancer stem cells. To confirm that AMPK activation was dose

Table 1. FNDs with substituents X or Y in the "first" aryl ring and substituents A,B,C,D in the "second aryl" ring. AMPK activation ratios were relative to compound 3 from a previous study [13]

Compound	Structure	Formula	MW	AMPK activation ratio	X	Y	A	B	C	D
4a		C ₁₅ H ₁₀ F ₆ N ₂ O ₂	364.243	2.37	CF ₃ O	H			CF ₃	
4b		C ₁₅ H ₉ ClF ₆ N ₂ O ₂ S	430.753	2.60	CF ₃ O	H		Cl	SCF ₃	
4c		C ₁₅ H ₁₀ F ₆ N ₂ OS	380.308	2.16	H	CF ₃ S			CF ₃	
4d		C ₁₄ H ₉ Cl ₂ F ₃ N ₂ OS	381.200	1.94	H	CF ₃ S		Cl		Cl
4e		C ₁₄ H ₈ F ₆ N ₂ O	334.217	4.00	CF ₃	H		F	F	F
4f		C ₁₄ H ₈ F ₆ N ₂ OS	366.282	2.02	CF ₃ S	H		F	F	F
4g		C ₁₄ H ₈ Cl ₂ F ₃ N ₂ OS	415.645	2.32	CF ₃ S	H		Cl	Cl	Cl
4h		C ₁₄ H ₇ F ₇ N ₂ OS	384.272	2.48	CF ₃ S	H	F	F	F	F

responsive in colorectal cancer stem cells, we treated colorectal cancer stem cell line 1 with 5, 25, and 50 $\mu\text{mol/L}$ of FND 4b. Our results showed a dose-dependent AMPK activation after FND 4b treatment (Fig. 2B).

Next, we used compound 4b to evaluate dose-dependent AMPK activation in KM20 and HT29 cells. We observed AMPK activation starting with as little as 10 $\mu\text{mol/L}$ (Fig. 3A). We also examined the dose dependent effects of FND 4b treatment on cell viability (Fig. 3B), cell cycle, and apoptosis induction in KM20 and HT29 cells. KM20 cells were more sensitive to compound 4b treatment and showed marked suppression of cyclin D1 expres-

sion at a dose of 10 $\mu\text{mol/L}$. PARP cleavage was detected in both cell lines starting at a 40 $\mu\text{mol/L}$ dose.

Treatment with FND 4b induces cell-cycle inhibition and apoptosis in *pik3ca* mutant colorectal cancer cells

We evaluated whether the *pik3ca* mutation protects cancer cells against FND 4b treatment. HCT116 *pik3ca* mutant and wild-type colorectal cancer cell lines were treated with FND 4b at 10, 20, 30, 40, and 50 $\mu\text{mol/L}$ for 24 hours. Both cell lines showed strong cell-cycle inhibition but required higher dosages compared to KM20 and HT29 cell lines. PARP cleavage was noted with FND 4b treatment at 20 $\mu\text{mol/L}$ (Fig. 4A). These results show that the HCT116 *pik3ca* mutant and wild-type cells have a similar pattern of cell-cycle inhibition and apoptosis in response to FND 4b treatment.

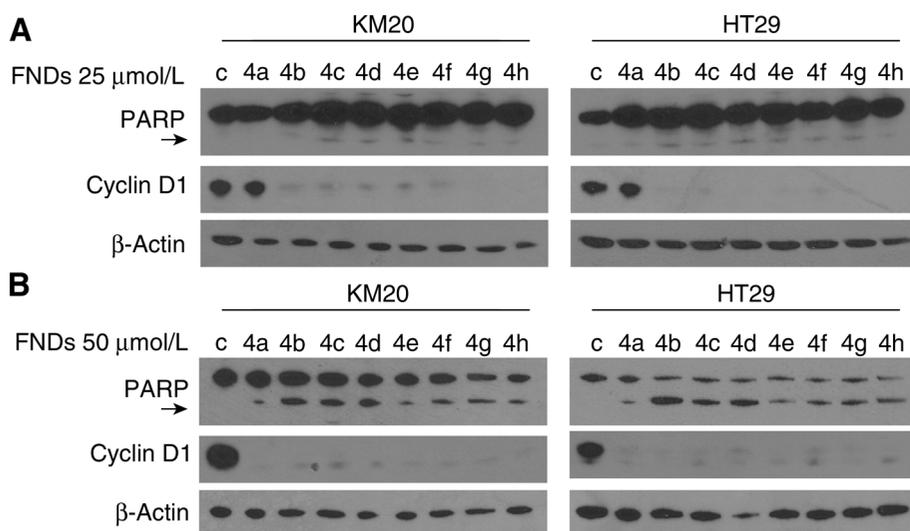
Metformin, which is a known activator of AMPK and is used for diabetes treatment and cancer prevention, activates AMPK at millimolar levels (15). AMPK downregulates TOR/S6K activity via TSC2 in response to stress (16). We treated HT29 and KM20 cells with FND 4b at 10, 20, and 30 $\mu\text{mol/L}$ and with metformin at 5, 10, and 20 mmol/L for 24 hours to compare the effect on cyclin D1 expression and S6 phosphorylation at Ser234/235. Both metformin and FND 4b decreased phosphorylation of the S6 protein, a downstream effector of mTOR that is a crucial signaling pathway for proliferation and survival of cancer cells *in vitro* as well as tumors *in vivo* (Fig. 4B and C). Our results demonstrated that FND 4b had a similar effect on cell-cycle inhibition and inhibition of S6 phosphorylation at the 20 $\mu\text{mol/L}$ dose as compared to the 10 mmol/L dose of metformin.

Table 2. Effect of FNDs on metastatic colorectal cancer cell viability

Compound	Control	5 $\mu\text{mol/L}$	10 $\mu\text{mol/L}$	25 $\mu\text{mol/L}$
4a	100	67.7 ^a	23.1 ^a	15.5 ^a
4b	100	36.0 ^a	22.2 ^a	8.3 ^a
4c	100	21.4 ^a	21.0 ^{aa}	6.6 ^a
4d	100	24.1 ^a	19.3 ^a	4.8 ^a
4e	100	29.9 ^a	23.6 ^a	9.7 ^a
4f	100	20.9 ^a	21.0 ^a	5.1 ^a
4g	100	22.6 ^a	23.2 ^a	7.5 ^a
4h	100	23.2 ^a	20.0 ^a	6.3 ^a

NOTE: HT29 cells were seeded in 96-well plates in complete medium (5×10^3 /well) and treated with 8 different FNDs (4a-4h). DMSO (25 $\mu\text{mol/L}$) was used as a treatment control. Cell viability was measured using a sulforhodamine B assay (SRB) assay after 48 hours of FND exposure and plotted as a percentage relative to DMSO treatment alone. Each measurement was performed in sextuplicate.

^a $P \leq 0.05$.

**Figure 1.**

Effect of FNDs on cyclin D1 expression and PARP cleavage in metastatic colorectal cancer cell lines. **A** and **B**, KM20 and HT29 colorectal cancer cells were seeded in 6-well plates (8×10^5 /well) in complete medium and treated 24 hours later with eight different FNDs (4a–4h) for 24 hours at 25 $\mu\text{mol/L}$ (**A**) and 50 $\mu\text{mol/L}$ (**B**). Cyclin D1 expression and PARP cleavage (PARP cleaved product 85 kDa; arrow) was assessed by Western blot analysis; β -actin was used as a loading control.

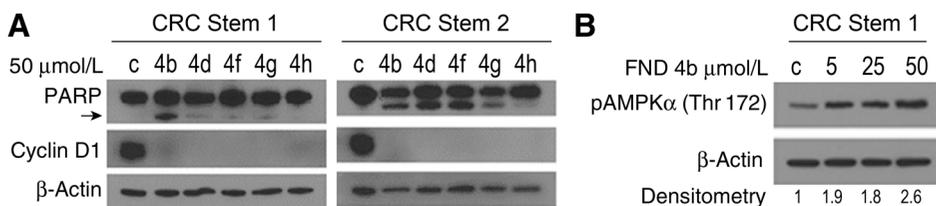
Treatment of colorectal cancer cells, stem cells, and PDX cell line with low-dose FND 4b

To determine the effect of FND treatment on AMPK α activation, HT29 cells were treated with low dosages (i.e., 5 and 10 μM) of FND 4b for 12, 24, 48, and 72 hours. FND treatment demonstrated a long-lasting effect on AMPK activation at 10 $\mu\text{mol/L}$ (Fig. 5A). Low-dose FND 4b treatment resulted in a strong AMPK α activation, and decreased cyclin D1 expression in colorectal cancer stem cells at 48 hours (Fig. 5B and C). Next, we established a cell line from a patient-derived xenograft (PDX) and treated it with low-dose FND 4b for 48 hours. Cyclin D1 expression was markedly suppressed in the PDX cell line (Fig. 5D). FND treatment at 5, 7.5, and 10 $\mu\text{mol/L}$ significantly decreased stem cells and PDXL proliferation (Fig. 5 B–D, bottom). Low-dose FND treatment did not increase PARP cleavage in all tested cells (data not shown). These results demonstrate a long-lasting effect of low-dose FND 4b treatment on AMPK α activation and cell-cycle inhibition.

Discussion

The central regulatory role played by AMPK in energy homeostasis makes it an attractive target for treatment of various diseases

such as diabetes and cancer (6, 7, 17). Recent efforts have focused on elucidating the complex role of AMPK in cancer cell viability (18). We examined the effects of eight fluorinated N,N'-diarylureas as AMPK activators on colorectal cancer metastatic and stem cell lines to evaluate their potential utility as chemotherapeutic agents for colorectal cancer. The FNDs that were used in this study represent a new class of AMPK activators with no effect on AKT or ERK, unlike other N,N' diarylureas [12]. We confirmed that FND treatment of colorectal cancer metastatic and stem cell lines resulted in dose-dependent AMPK activation. High doses of traditional AMPK activators, such as AICAR and metformin, were required to achieve anticancer effects (10–12, 19). However, similar to FNDs, more recent AMPK activators showed antitumorogenic effects at lower concentrations. Chen and colleagues (19) showed that plumbagin, an AMPK activator, induced apoptosis and inhibited growth of colorectal cancer cell lines at a treatment concentration of 10 $\mu\text{mol/L}$. Kaushik and colleagues (20) showed that honokiol, another AMPK activating compound, inhibited melanoma growth and spheroid-forming capacity (i.e., stemness) through AMPK activation at concentrations up to 50 $\mu\text{mol/L}$. Finally, Valtorta and colleagues (21) showed that one to four diaryl-azetidinone, also a novel AMPK activator, inhibited human colorectal cancer xenografts in mouse models

**Figure 2.**

Effect of FNDs on PARP cleavage and cyclin D1 and pAMPK expression in colorectal cancer stem cell lines. **A**, colorectal cancer stem cell line 1 and stem cell line 2 were seeded in 6-well plates in complete medium and treated 24 hours later with FNDs 4b, 4d, 4f, 4g, and 4h at 50 $\mu\text{mol/L}$ for 24 hours. Cyclin D1 expression and PARP cleavage (PARP cleaved product 85 kDa; arrow) was analyzed by Western blot analysis. DMSO (50 $\mu\text{mol/L}$) was used as a treatment control, and β -actin was used as a loading control. **B**, colorectal cancer stem cell line 1 was seeded in 6-well plates (8×10^5 /well) in complete medium and treated 24 hours later with FND 4b at 5, 25, and 50 $\mu\text{mol/L}$ for 12 hours. AMPK α phosphorylation (Thr172) was analyzed by Western blot analysis. DMSO (50 $\mu\text{mol/L}$) was used as a treatment control, and β -actin was used as a loading control. Densitometry was performed with ImageJ software; relative pAMPK α (Thr172) band intensity was normalized to β -actin and quantified with respect to control set at 1.0.

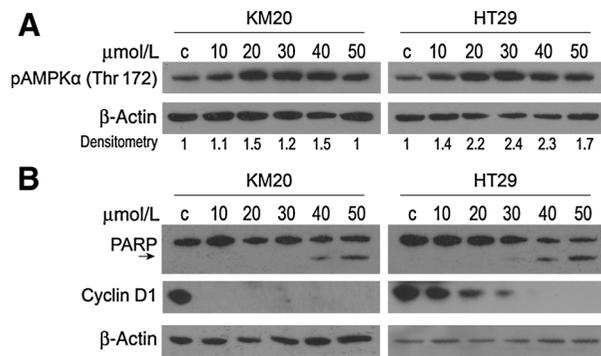


Figure 3.

Treatment of colorectal cancer cell lines with FND 4b. **A**, KM20 and HT29 colorectal cancer cells were seeded in 6-well plates (8×10^5 /well) in complete medium and treated 24 hours later with FND 4b at 10, 20, 30, 40, and 50 $\mu\text{mol/L}$ for 12 hours. AMPK α phosphorylation (Thr172) was assessed by Western blot analysis. DMSO (50 $\mu\text{mol/L}$) was used as a treatment control, and β -actin was used as a loading control. Densitometry was performed with ImageJ software; relative pAMPK α (Thr172) band intensity was normalized to β -actin and quantified with respect to control set at 1.0. **B**, KM20 and HT29 colorectal cancer cells were seeded in 6-well plates (8×10^5 /well) in complete medium for 24 hours and treated with FND 4b at 10, 20, 30, 40, and 50 $\mu\text{mol/L}$ for 24 hours. Cyclin D1 expression and PARP cleavage (cleaved product 85 kDa; arrow) was assessed by Western blot analysis. DMSO (50 $\mu\text{mol/L}$) was used as a treatment control, and β -actin was used as a loading control.

at IC_{50} in the nmol/L to 1 $\mu\text{mol/L}$ range. Taken together, these studies suggested that targeting cancer cells with novel AMPK activators represent an effective strategy for the treatment of

different types of cancers. Thus, the beneficial effects on metastatic cancer cells by AMPK activation provide a rationale for further studies, to determine the potential clinical utility of AMPK activators, like FNDs, on cancer stem cells.

In our study, we evaluated the effect of FNDs on colorectal cancer stem cells. Many cancer stem cell models have been described, but a unifying theme in tumorigenesis is that tumors are not homogeneous. A hierarchical structure within tumors includes a small subset of cells with long-term self-renewal and differentiation capacity (3). Colorectal cancer, in particular, is known to possess cancer stem cells (22). To survive, repopulate, and differentiate, cancer stem cells have developed several mechanisms that provide resistance to the effects of antineoplastic agents. These mechanisms include resistance to DNA damage, drug penetration, apoptosis, and reactive oxygen species (ROS; refs. 3–5). Upregulation of the DNA repair machinery and down-regulation of telomerase allow cancer stem cells to maintain their genetic integrity. Cancer stem cells also upregulate efflux transporters from the ATP-binding cassette (ABC) gene family to efficiently extrude chemotherapy agents. Resistance to apoptosis occurs, in part, through Akt pathway activation and increased production of apoptosis inhibitor proteins. Finally, cancer stem cells increase their free radical scavenging machinery to protect themselves from ROS damage (4). Several studies have investigated the role of stem cells in treatment resistance (2, 11). For example, Zhou and colleagues (2) created cisplatin-resistant colorectal cancer cells and found that these cells had stem cell-like markers. Treatment with salinomycin caused an accumulation of ROS and down-regulation of antiapoptotic molecules, which suggested that salinomycin was able to overcome the cisplatin resistance of colorectal cancer cells. Because cancer

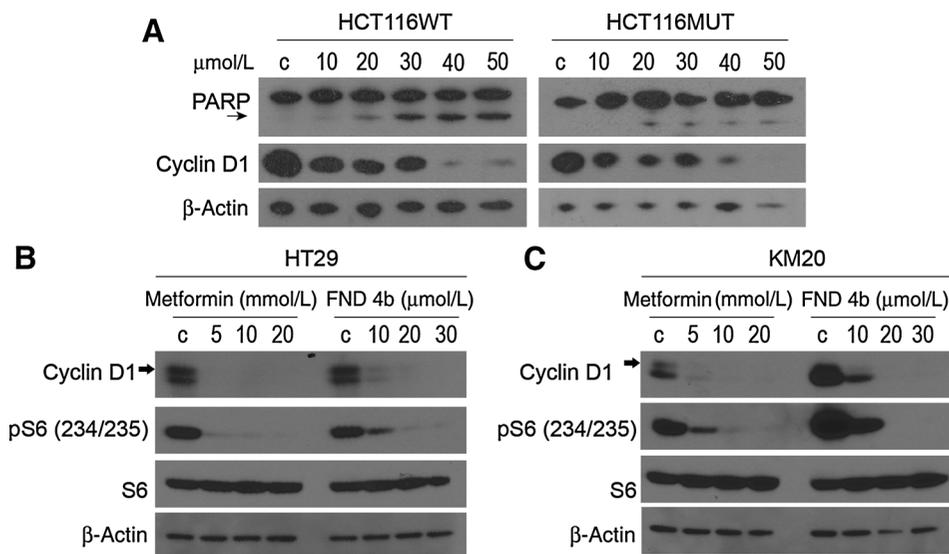


Figure 4.

Treatment of *Pik3ca* mutant colorectal cancer cells with FND 4b. **A**, HCT116 *pik3ca* wild-type and *pik3ca* mutant cell lines were seeded in 6-well plates (8×10^5 /well) in complete medium for 24 hours and treated with FND 4b at 10, 20, 30, 40, and 50 $\mu\text{mol/L}$ for 24 hours. Cyclin D1 expression and PARP cleavage (PARP cleaved product 85 kDa; arrow) was analyzed by Western blot analysis. DMSO (50 $\mu\text{mol/L}$) was used as a treatment control; β -actin was used as a loading control. **B** and **C**, HT29 and KM20, respectively, cells were seeded in 24-well plates (5×10^3 /well) in complete medium and treated 24 hours later with 5, 10, 20 mmol/L metformin and FND 4b at 10, 20, 30 $\mu\text{mol/L}$ for 24 hours. Cyclin D1 (arrow), S6 phosphorylation (Ser234/235), and total S6 expression was analyzed by Western blot analysis. DMSO (30 $\mu\text{mol/L}$) was used as a treatment control; β -actin was used as a loading control.

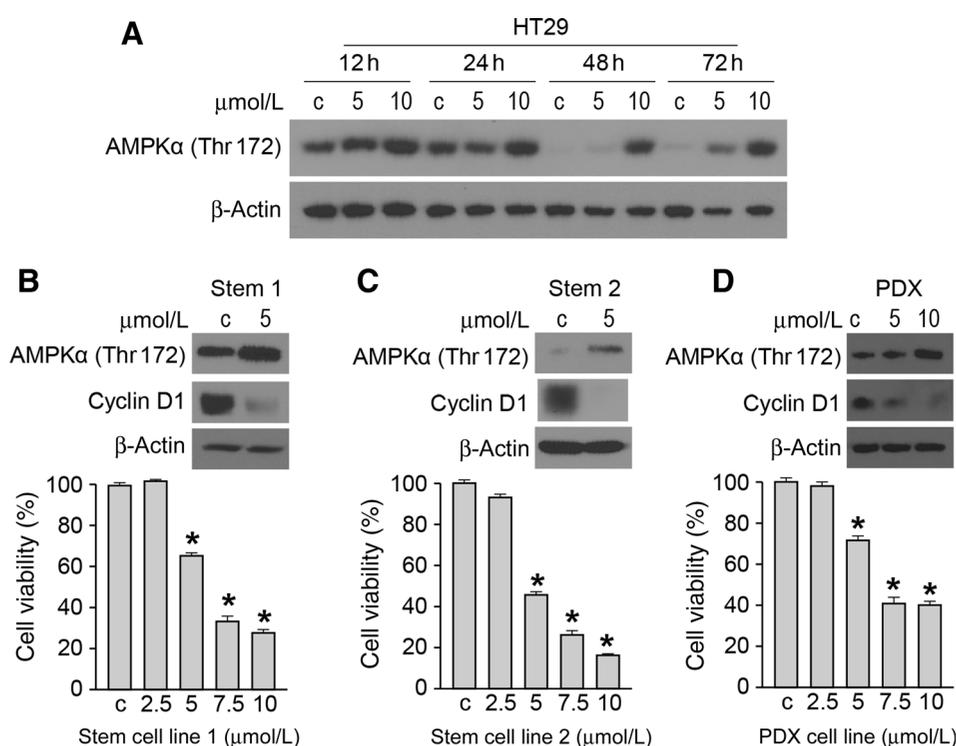


Figure 5.

Low-dose FND 4b treatment. **A**, HT29 colorectal cancer cells were seeded in 6-well plates (8×10^5 /well) in complete medium for 24 hours and treated with FND 4b at 5 and 10 $\mu\text{mol/L}$ for 12, 24, 48, and 72 hours. AMPK α phosphorylation (Thr172) was analyzed by Western blot analysis. DMSO (10 $\mu\text{mol/L}$) was used as a treatment control; β -actin was used as a loading control. **B** and **C** (top), colorectal cancer stem cell line 1 and stem cell line 2 were seeded in 6-well plates in complete medium and treated 24 hours later with 5 $\mu\text{mol/L}$ FND 4b for 48 hours. AMPK α phosphorylation (Thr172) and cyclin D1 expression were analyzed by Western blot analysis. DMSO (10 $\mu\text{mol/L}$) was used as a treatment control; β -actin was used as a loading control; (bottom) stem cells were seeded in 96-well plates in complete medium (5×10^3 /well) and treated with FND 4b. Cell viability (bottom) was measured using an SRB assay after 48 hours of FND exposure and plotted as a percentage relative to DMSO treatment alone. Graphic representations are the mean \pm SE; each measurement was performed in sextuplicate. **D**, PDX cell line was seeded in 6-well plates (8×10^5 /well) in complete medium and treated 24 hours later with FND 4b at 5 and 10 $\mu\text{mol/L}$ for 48 hours. Cyclin D1 expression was analyzed by Western blot analysis (top). DMSO (10 $\mu\text{mol/L}$) was used as a treatment control; β -actin was used as a loading control. PDX cell line was seeded in 96-well plates in complete medium (5×10^3 /well) and treated with FND 4b. Cell viability (bottom) was measured using an SRB assay after 48 hours of FND exposure and plotted as a percentage relative to DMSO treatment alone. Graphic representations are the mean \pm SE; each measurement was performed in sextuplicate; *, $P \leq 0.05$.

stem cells possess these survival mechanisms, cancer treatment plans should include therapy to specifically target cancer stem cells in order to reduce future recurrence and metastasis.

Although the specific role of AMPK in cancer stem cells compared with the non-stem cell population has not yet been fully characterized, several studies have shown that metformin, an AMPK activator, is preferentially cytotoxic to cancer stem cells in pancreatic and breast cancers (10, 11, 23). Similarly, in our present study, we showed that FND treatment resulted in cell-cycle arrest in colorectal cancer stem cells. AMPK activation led to inhibition of macromolecular synthesis, inhibition of mTORC1, and onset of the "anti-Warburg effect" (7, 24, 25), all of which led to the inhibition of cancer cell proliferation. Hardie and Alessi (7) described several AMPK tumor suppressor functions that included cell-cycle arrest through stabilization of p53, p21, and p27. Particularly in cancer stem cells, it is likely that the anti-Warburg effect targets the utilization of anaerobic glycolysis by cancer stem cells, and decreases their ability to use AKT-mTOR. AMPK activation of apoptosis resulted from p53 phosphorylation; phosphorylated AMPK subsequently accumulated in the mitochondria and interacted with Bak to induce apoptosis (8). Our results

demonstrated that AMPK activation reduced the ability of cancer stem cells to hijack energy metabolism for growth and proliferation and triggered an apoptotic cascade. In summary, we demonstrated that FNDs activated AMPK at micromolar concentrations, which resulted in consistent cell-cycle arrest and apoptosis in all cancer cell lines that were studied. These findings provide additional information regarding the role of AMPK activation in cancer stem cells and demonstrate a potential role of FNDs in the treatment of metastatic colorectal cancer.

Disclosure of Potential Conflicts of Interest

C. Liu has ownership interest (including patents) in Liu-Watt, LLC. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: P. Rychahou, C. Liu, D.S. Watt
 Development of methodology: P. Rychahou, D.S. Watt
 Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): D.E. Kenlan, V.M. Sviripa, D.S. Watt
 Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): D.E. Kenlan, V.M. Sviripa, H.L. Weiss, D.S. Watt

Writing, review, and/or revision of the manuscript: D.E. Kenlan, P. Rychahou, V.M. Sviripa, C. Liu, D.S. Watt, B.M. Evers

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): D.E. Kenlan, D.S. Watt

Study supervision : P. Rychahou, D.S. Watt, B.M. Evers

Acknowledgments

The authors thank the Markey Cancer Center's Research Communications Office for help with manuscript preparation, and the Biostatistics and Bioinformatics Shared Resource Facility for statistical analysis. The contents of this manuscript are solely the responsibility of the authors and do not necessarily represent the official views of the grant funding agencies.

References

- Siegel R, Ma J, Zou Z, Jemal A. Cancer statistics, 2014. *CA Cancer J Clin* 2014;64:9–29.
- Zhou J, Li P, Xue X, He S, Kuang Y, Zhao H, et al. Salinomycin induces apoptosis in cisplatin-resistant colorectal cancer cells by accumulation of reactive oxygen species. *Toxicol Lett* 2013;222:139–45.
- Beck B, Blanpain C. Unravelling cancer stem cell potential. *Nat Rev Cancer* 2013;13:727–38.
- Morrison R, Schleicher SM, Sun Y, Niermann KJ, Kim S, Spratt DE, et al. Targeting the mechanisms of resistance to chemotherapy and radiotherapy with the cancer stem cell hypothesis. *J Oncol* 2011;2011:941876.
- Ito K, Suda T. Metabolic requirements for the maintenance of self-renewing stem cells. *Nat Rev Mol Cell Biol* 2014;15:243–56.
- Mihaylova MM, Shaw RJ. The AMPK signalling pathway coordinates cell growth, autophagy and metabolism. *Nat Cell Biol* 2011;13:1016–23.
- Hardie DG, Alessi DR. LKB1 and AMPK and the cancer-metabolism link - ten years after. *BMC Biol* 2013;11:36.
- Nieminen AI, Eskelinen VM, Haikala HM, Tervonen TA, Yan Y, Partanen JJ, et al. Myc-induced AMPK-phospho p53 pathway activates Bak to sensitize mitochondrial apoptosis. *Proc Natl Acad Sci U S A* 2013;110:E1839–48.
- Sehdev A, Shih YC, Vekhter B, Bissonnette MB, Olopade OI, Polite BN. Metformin for primary colorectal cancer prevention in patients with diabetes: a case-control study in a US population. *Cancer* 2015;121:1071–8.
- Hirsch HA, Iliopoulos D, Tschlis PN, Struhl K. Metformin selectively targets cancer stem cells, and acts together with chemotherapy to block tumor growth and prolong remission. *Cancer Res* 2009;69:7507–11.
- Song CW, Lee H, Dings RP, Williams B, Powers J, Santos TD, et al. Metformin kills and radiosensitizes cancer cells and preferentially kills cancer stem cells. *Sci Rep* 2012;2:362.
- Sviripa V, Zhang W, Conroy MD, Schmidt ES, Liu AX, Truong J, et al. Fluorinated N,N'-diarylureas as AMPK activators. *Bioorg Med Chem Lett* 2013;23:1600–3.
- Gulhati P, Bowen KA, Liu J, Stevens PD, Rychahou PG, Chen M, et al. mTORC1 and mTORC2 regulate EMT, motility, and metastasis of colorectal cancer via RhoA and Rac1 signaling pathways. *Cancer Res* 2011;71:3246–56.
- Guo XN, Rajput A, Rose R, Hauser J, Beko A, Kuropatwinski K, et al. Mutant PIK3CA-bearing colon cancer cells display increased metastasis in an orthotopic model. *Cancer Res* 2007;67:5851–8.
- Zhou G, Myers R, Li Y, Chen Y, Shen X, Fenyk-Melody J, et al. Role of AMP-activated protein kinase in mechanism of metformin action. *J Clin Invest* 2001;108:1167–74.
- Inoki K, Zhu T, Guan KL. TSC2 mediates cellular energy response to control cell growth and survival. *Cell* 2003;115:577–90.
- Fay JR, Steele V, Crowell JA. Energy homeostasis and cancer prevention: the AMP-activated protein kinase. *Cancer Prev Res* 2009;2:301–9.
- Liang J, Mills GB. AMPK: a contextual oncogene or tumor suppressor? *Cancer Res* 2013;73:2929–35.
- Chen MB, Zhang Y, Wei MX, Shen W, Wu XY, Yao C, et al. Activation of AMP-activated protein kinase (AMPK) mediates plumbagin-induced apoptosis and growth inhibition in cultured human colon cancer cells. *Cell Signal* 2013;25:1993–2002.
- Kaushik G, Kwatra D, Subramaniam D, Jensen RA, Anant S, Mammen JM. Honokiol affects melanoma cell growth by targeting the AMP-activated protein kinase signaling pathway. *Am J Surg* 2014;208:995–1002.
- Valtorta S, Nicolini G, Tripodi F, Meregalli C, Cavaletti G, Avezza F, et al. A novel AMPK activator reduces glucose uptake and inhibits tumor progression in a mouse xenograft model of colorectal cancer. *Invest New Drugs* 2014;32:1123–33.
- Fanali C, Luchetti D, Farina M, Corbi M, Cufino V, Cittadini A, et al. Cancer stem cells in colorectal cancer from pathogenesis to therapy: controversies and perspectives. *World J Gastroenterol* 2014;20:923–42.
- Fasih A, Elbaz HA, Huttemann M, Konski AA, Zielske SP. Radiosensitization of pancreatic cancer cells by metformin through the AMPK pathway. *Radiat Res* 2014;182:50–9.
- Faubert B, Boily G, Izreig S, Griss T, Samborska B, Dong Z, et al. AMPK is a negative regulator of the Warburg effect and suppresses tumor growth in vivo. *Cell Metab* 2013;17:113–24.
- Shackelford DB, Shaw RJ. The LKB1-AMPK pathway: metabolism and growth control in tumour suppression. *Nat Rev Cancer* 2009;9:563–75.