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## Induction of AMPK Activation by *N,N'*-Diarylurea FND-4b Decreases Growth and Increases Apoptosis in Triple Negative and Estrogen-Receptor Positive Breast Cancers

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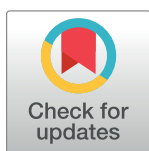
RESEARCH ARTICLE

# Induction of AMPK activation by *N,N'*-diaryurea FND-4b decreases growth and increases apoptosis in triple negative and estrogen-receptor positive breast cancers

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## Abstract

### Purpose

Triple negative breast cancer (TNBC) is the most lethal and aggressive subtype of breast cancer. AMP-activated protein kinase (AMPK) is a major energy regulator that suppresses tumor growth, and 1-(3-chloro-4-((trifluoromethyl)thio)phenyl)-3-(4-(trifluoromethoxy)phenyl)urea (FND-4b) is a novel AMPK activator that inhibits growth and induces apoptosis in colon cancer. The purpose of this project was to test the effects of FND-4b on AMPK activation, proliferation, and apoptosis in breast cancer with a particular emphasis on TNBC.

### Materials and methods

(i) Estrogen-receptor positive breast cancer (ER+BC; MCF-7, and T-47D), TNBC (MDA-MB-231 and HCC-1806), and breast cancer stem cells were treated with FND-4b for 24h. Immunoblot analysis assessed AMPK, acetyl-CoA carboxylase (ACC), ribosomal protein S6, cyclin D1, and cleaved PARP. (ii) Sulforhodamine B growth assays were performed after treating ER+BC and TNBC cells with FND-4b for 72h. Proliferation was also assessed by counting cells after 72h of FND-4b treatment. (iii) Cell death ELISA assays were performed after treating ER+BC and TNBC cells with FND-4b for 72h.

### Results

(i) FND-4b increased AMPK activation with concomitant decreases in ACC activity, phosphorylated S6, and cyclin D1 in all subtypes. (ii) FND-4b decreased proliferation in all cells, while dose-dependent growth decreases were found in ER+BC and TNBC. (iii) Increases in apoptosis were observed in ER+BC and the MDA-MB-231 cell line with FND-4b treatment.

**Competing interests:** CL and DSW have partial ownership in a private venture, Epionc Inc., incorporated to develop small-molecule inhibitors for cancer treatment. In accord with University of Kentucky policies, CL and DSW have disclosed this work to the University of Kentucky's Intellectual Property Committee and complied with stipulations of the University's Conflict of Interest Oversight Committee. This does not alter our adherence to PLOS ONE policies on sharing data and materials.

## Conclusions

Our findings indicate that FND-4b decreases proliferation for a variety of breast cancers by activating AMPK and has notable effects on TNBC. The growth reductions were mediated through decreases in fatty acid synthesis (ACC), mTOR signaling (S6), and cell cycle flux (cyclin D1). ER+BC cells were more susceptible to FND-4b-induced apoptosis, but MDA-MB-231 cells still underwent apoptosis with higher dose treatment. Further development of FND compounds could result in a novel therapeutic for TNBC.

## Introduction

Breast cancer is the most common cancer in women and the main cause of cancer-related death among women worldwide. In 2018 alone, there will be more than 266,000 newly diagnosed cases of breast cancer in women in the United States and almost 41,000 deaths [1]. Up to 30% of patients develop metastases, and 90% of deaths result from metastases to the lung, brain, or bone [2]. Breast cancer is a heterogeneous disease separable into three main types: estrogen-receptor positive breast cancer (ER+BC), HER2-amplified breast cancer, and triple negative breast cancer (TNBC). Although TNBC comprises only 15–20% of total cases, it is the most lethal and aggressive of the three types [3, 4].

The principal characteristics of TNBC include: (1) reduced expression of the estrogen and progesterone receptors and (2) no overexpression of HER2. TNBC affects a younger patient population than the population afflicted with other types of breast cancer and leads to an increased risk of recurrence and metastases [3]. Not surprisingly, patients with recurrent TNBC have a worse prognosis than that for patients with recurrent forms of other breast cancers [3]. In addition, patients with TNBC have limited therapeutic options because their tumors lack the traditional steroid hormone receptors and HER2 amplification. Instead, patients usually receive a drug cocktail that includes an anthracycline antineoplastic agent, a DNA alkylating agent, and a taxane [3]. These chemotherapeutic agents are toxic to normal and cancer cells alike and result in serious side-effects that are difficult for patients to tolerate. Recent efforts have focused on developing therapies that specifically target cancer cells without affecting normal cells. Because oncogenic transformation requires major metabolic reprogramming to produce energy, redox cofactors, and molecules involved in DNA modification, new agents that target the increased metabolism within cancer tissue more than the metabolism in normal tissue are attractive therapeutic options [2].

AMP-activated protein kinase (AMPK) is a cellular energy sensor that has important implications in cancer progression [5–16]. When activated by ATP depletion, the phosphorylated form of AMPK causes the following changes in TNBC: (1) inhibition of anabolic and oncogenic pathways, (2) attenuated mTOR signaling, (3) decreased cell proliferation, and (4) apoptosis [17–26]. Well-known AMPK activators, such as 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) and 2-deoxyglucose (2-DG), require high doses to affect cancer cell proliferation, which has led to their unsuccessful translation to the clinic for cancer therapy [12]. Among the attempts to produce new AMPK activators with increased sensitivity, the fluorinated *N,N'*-diarylsureas (FNDs) serve as activators that lead to phosphorylated AMPK at low concentrations [27, 28]. In particular, 1-(3-chloro-4-((trifluoromethyl)thio)phenyl)-3-(4-(trifluoromethoxy)phenyl)urea (FND-4b) inhibits growth and induces apoptosis in colorectal cancer cells, but its potential effects on other types of cancer remain unclear [27]. Because of

the pressing need to develop new treatments for TNBC, we tested the effects of FND-4b on TNBC and compared the results with ER+BC. Importantly, we found that treatment with FND-4b led to AMPK activation, decreased cell cycle flux, and increased apoptosis in both subtypes. These findings indicate that FND compounds may be potential therapeutic options for TNBC.

## Materials and methods

### Reagents, supplements, and antibodies

1-(3-Chloro-4-((trifluoromethyl)thio)phenyl)-3-(4-(trifluoromethoxy)phenyl)urea (FND-4b) was synthesized and characterized as previously described [28]. Roswell Park Memorial Institute (RPMI) 1640 Medium and Eagle's Minimum Essential Medium (EMEM) were purchased from Sigma-Aldrich (St. Louis, MO). Dulbecco's Modified Eagle Medium (DMEM) was from Corning (Corning, NY). Human breast cancer stem cell complete growth medium was from Celprogen (Torrance, CA). MEM non-essential amino acid solution (100x), sodium pyruvate solution (100 mM), insulin solution (10 mg/mL), penicillin-streptomycin (100x) (P/S), and fetal bovine serum (FBS) were from Sigma-Aldrich. 5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR) was from Abcam (Cambridge, MA). Antibodies for pAMPK $\alpha$  (Thr172), total AMPK $\alpha$ , phosphorylated acetyl-CoA carboxylase (ACC), total ACC, phosphorylated ribosomal protein S6, total S6, and PARP were from Cell Signaling Technology (CST; Danvers, MA). The cyclin D1 antibody was from Abcam. The beta-actin antibody was from Sigma-Aldrich. The secondary antibodies to rabbit and mouse were from Santa-Cruz. All relevant antibody information is in Table 1. The Sulforhodamine B (SRB) Cytotoxicity Assay was from G-Biosciences (St. Louis, MO). The Cell Death Detection ELISA<sup>PLUS</sup> assay was from Sigma-Aldrich.

### Cell culture

MCF-7, T-47D, MDA-MB-231, HCC-1143, and HCC-1806 cells were purchased from ATCC, while breast cancer stem cells were purchased from Celprogen. MCF-7 cells were maintained in EMEM containing 10% FBS, 1% P/S, 0.01 mg/mL insulin, 1x non-essential amino acids, and 1 mM sodium pyruvate. T-47D cells were maintained in RPMI containing 10% FBS, 1% P/S, and 0.2 Units/mL insulin. MDA-MB-231, HCC-1143, and HCC-1806 cells were maintained in RPMI with 10% FBS and 1% P/S. Breast cancer stem cells were maintained in breast cancer stem cell medium supplemented with 10% FBS and 1% P/S. All cells were grown in an

**Table 1. Primary and secondary antibody information.**

Antibody	Monoclonal vs. Polyclonal	Host Species	Dilution	Supplier	Catalogue Number
pAMPK $\alpha$	Monoclonal	Rabbit	1:500	CST	2535
Total AMPK $\alpha$	Polyclonal	Rabbit	1:1000	CST	2532
pACC	Polyclonal	Rabbit	1:1000	CST	3661
Total ACC	Monoclonal	Rabbit	1:1000	CST	3676
pS6	Polyclonal	Rabbit	1:1000	CST	2211
Total S6	Monoclonal	Rabbit	1:1000	CST	2217
Cyclin D1	Monoclonal	Rabbit	1:2500	Abcam	ab134175
PARP	Monoclonal	Rabbit	1:1000	CST	9532
Beta-actin	Monoclonal	Mouse	1:10000	Sigma-Aldrich	A1978
Rabbit	-	Goat	1:10000	Santa Cruz	sc-2054
Mouse	-	Goat	1:10000	Santa Cruz	sc-2055

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incubator at 37°C and 5% CO<sub>2</sub>. For cell treatments, 7x10<sup>5</sup> MCF-7 and T-47D cells or 8x10<sup>5</sup> MDA-MB-231, HCC-1806, and breast cancer stem cells were seeded in 6-well plates and incubated overnight. The medium was removed on the following day, and cells were treated with fresh medium that contained different concentrations of FND-4b (0, 1, 2.5, 5, 10, and 20 μM) for 24 h before lysis.

### Western blot analysis

After treatment, cells were scraped from the wells with 1x RIPA buffer containing serine protease inhibitor. The cells were lysed by incubating on ice for 20 min and vortexing 10 sec every 5 min. The lysates were centrifuged at 14,000 rpm and 4°C for 20 min, and the protein concentration was determined using the Bradford method. The proteins were reduced and denatured by heating at 80°C for 10 min. An equal amount of protein was resolved on SDS-PAGE gels and transferred to PVDF membranes. The membranes were blocked with 10% milk before overnight incubation with primary antibodies at 4°C. On the following day, the membranes were washed with Tris buffered saline with 0.1% tween-20 (TBST) for 5 min and again for 10 min. The membranes were subsequently incubated with the appropriate secondary antibody for 30 min at room temperature. The membranes were then washed with TBST for 15 min and again for 20 min. Proteins were visualized with Amersham ECL (GE Healthcare) or Immobilon (Millipore). Membranes were stripped and reprobed as necessary.

### Cell counting assay

Cells (1x10<sup>5</sup>) were seeded in 6-well plates and incubated overnight. The medium was removed on the following day, and cells were grown in fresh medium that contained either: (a) 0 or 5 μM FND-4b or (b) 0 or 1 mM AICAR for 72 h. Cells were then washed with PBS, trypsinized, and counted with a Beckman-Coulter cell counter.

### SRB growth assay

Cells (5x10<sup>3</sup> in 100 μL) were seeded in 96-well plates and incubated overnight. On the following day, fresh media was prepared to contain twice the desired concentrations of FND-4b (i.e., 5, 10, 20, and 40 μM). Then 100 μL of the new media solutions were added to the wells without removing the old media; this yielded 200 μL per well and halved the FND-4b concentrations. Cells were grown in the media with different final concentrations of FND-4b (0, 2.5, 5, 10, and 20 μM) for 72 h before the proteins were fixed at 4°C for 1 h. The wells were washed 3 times with water and then dried for 30 min. The SRB dye solution (0.4%) was added to the wells and incubated for 30 min. The excess dye was washed off with 1% acetic acid, and the wells were allowed to air dry. The dye was solubilized with a 10 mM Tris solution, and the absorbance was measured at 565 nm or—if the readings were outside of the instrument's linear range—at 490 nm.

### Cell Death Detection ELISA<sup>PLUS</sup> assay

Cells (5x10<sup>3</sup> in 100 μL) were seeded in 96-well plates and incubated overnight. On the following day, fresh media was prepared to contain twice the desired concentrations of FND-4b (i.e., 5, 10, and 20 μM). Then 100 μL of the new media solutions were added to the wells without removing the old media; this yielded 200 μL per well and halved the FND-4b concentrations. Cells were cultured in the media with different final concentrations of FND-4b (0, 2.5, 5, and 10 μM) for 72 h. Cells were then centrifuged at 200 x g for 10 min and lysed for 30 min with shaking. The lysates were centrifuged at 200 x g for 10 min, and 20 μL of supernatant was

transferred to streptavidin-coated wells. Then 80  $\mu$ L of the immunoreagent (anti-histone biotin and anti-DNA peroxidase) was added to the streptavidin-coated wells. The plates were shaken for 2 h at room temperature before the wells were rinsed three times with incubation buffer. Color change was initiated by adding the substrate ABTS to the wells, and the plates were shaken until the color change was sufficient for photometric analysis. After adding the ABTS Stop Solution, absorbance was measured at 405 nm.

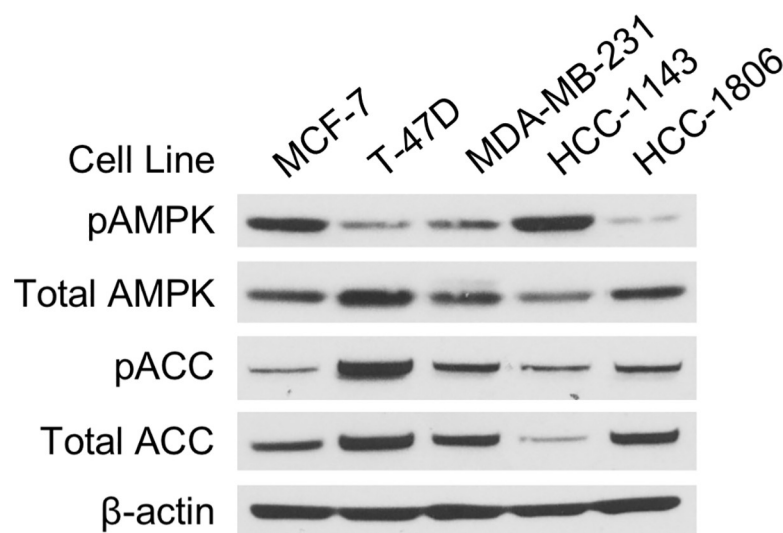
## Statistical analysis

Comparisons of SRB growth and ELISA assays across non-treated and different dose groups of FND-4b were performed using analysis of variance (ANOVA) with test for linear trend across dose levels. Pairwise comparisons of each FND-4b dose level versus non-treated group were performed within the ANOVA model with Holm's p-value adjustment for multiple testing. Comparisons of baseline ELISA values between HCC-1806 cells and other cell lines were performed using ANOVA with least squares means and adjustment for multiple comparisons. Comparisons of cell counting assays between control and FND-4b- or AICAR-treated groups were performed using two-sample t-tests with homogeneity of variance assessed for the use of the t-test for two group comparisons. Analyses were performed on data normalized with the non-treated group for the SRB growth and cell counting assays and on the raw data for the ELISA assays. In all experiments, p-values less than 0.05 were considered significant.

## Results

### Analysis of pAMPK $\alpha$ expression in breast cancer subtypes

Prior work has suggested that TNBC cell lines and tissues have higher levels of phosphorylated and total forms of AMPK $\alpha$  than non-TNBC cells and tissues [29]. Consequently, we compared levels of pAMPK $\alpha$  and total AMPK $\alpha$  in TNBC and ER+BC cells using western blotting. We found no difference in levels of phosphorylated or total AMPK $\alpha$  between two ER+BC cell lines (MCF-7 and T-47D) and three TNBC cell lines (MDA-MB-231, HCC-1143, and HCC-1806) (Fig 1).



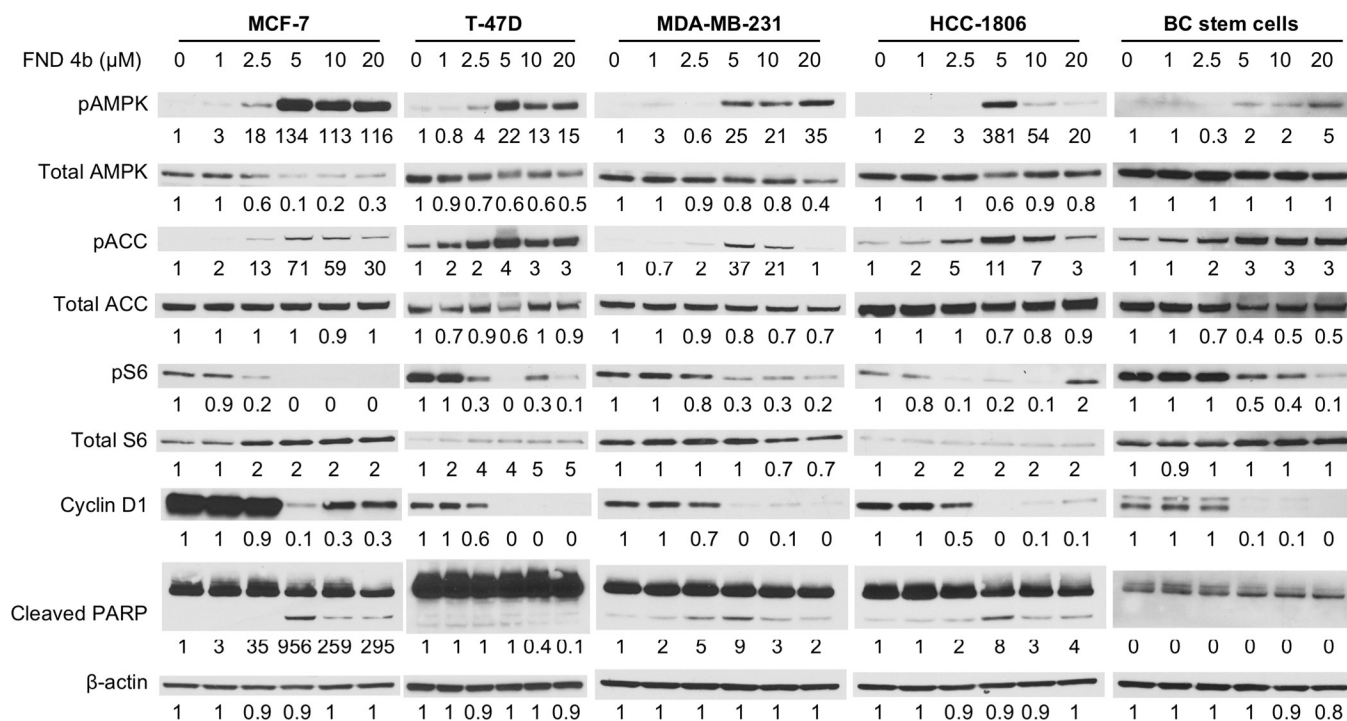
**Fig 1. Examination of pAMPK $\alpha$  levels in breast cancer subtypes.** Equal numbers of MCF-7, T-47D, HCC-1143, MDA-MB-231, and HCC-1806 cells were incubated overnight before cell lysis. Western blot analyses were performed for total and phosphorylated forms of AMPK $\alpha$  and ACC. Beta-actin was used as the loading control.

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## FND-4b activated AMPK $\alpha$ and downstream signaling pathways in breast cancer

Because FND-4b activated AMPK $\alpha$  in colon cancer cells, we investigated its effects in several subtypes of breast cancer [27]. In particular, MCF-7, T-47D, MDA-MB-231, HCC-1806, and breast cancer stem cells were treated with fresh medium containing a range of FND-4b concentrations (0, 1, 2.5, 5, 10, and 20  $\mu$ M) for 24 h. Protein levels of phosphorylated and total forms of AMPK $\alpha$ , acetyl-CoA carboxylase (ACC), and ribosomal protein S6, cyclin D1, and cleaved PARP were measured with immunoblotting. ACC and S6 were selected for analysis because they are downstream effectors of AMPK $\alpha$  that are phosphorylated and dephosphorylated, respectively, with AMPK $\alpha$  activation. As expected based on prior work, FND-4b treatment increased levels of pAMPK $\alpha$  in all five cell lines (Fig 2) [27]. Consistent with this result, increases in pACC and decreases in pS6 were also noted. Cyclin D1, an AMPK $\alpha$ -regulated marker for flux through the cell cycle, was decreased in all cell lines treated with FND-4b. Increases in the apoptotic indicator cleaved PARP were observed in MCF-7, MDA-MB-231, and HCC-1806 cells with AMPK $\alpha$  activation. FND-4b concentrations less than 5  $\mu$ M yielded minimal effects on AMPK signaling, but the 5  $\mu$ M dose yielded robust effects in all cells tested. The HCC-1806 cells were notable because AMPK $\alpha$  activation spiked with 5  $\mu$ M FND-4b treatment and then declined with concentrations higher than 5  $\mu$ M. Taken together, these results indicate that FND-4b activates AMPK $\alpha$  and its downstream signaling pathways in TNBC, ER+BC, and breast cancer stem cells.



**Fig 2. FND-4b activated AMPK $\alpha$  and its downstream signaling pathways in a dose-dependent manner in breast cancer.** Equal numbers of MCF-7, T-47D, MDA-MB-231, HCC-1806 or breast cancer stem cells were treated with fresh medium that contained different FND-4b concentrations (0, 1, 2.5, 5, 10, and 20  $\mu$ M) for 24 h. Western blot analysis was then performed for phosphorylated and total forms of AMPK $\alpha$ , ACC, and S6 as well as cyclin D1 and cleaved PARP. Beta-actin was used as the loading control. Treatment of the HCC-1806 cell line was repeated to confirm the spike in AMPK $\alpha$  activation at the 5  $\mu$ M dosage with subsequent decreases at higher concentrations.

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## FND-4b decreased growth of breast cancer subtypes in a dose-dependent fashion

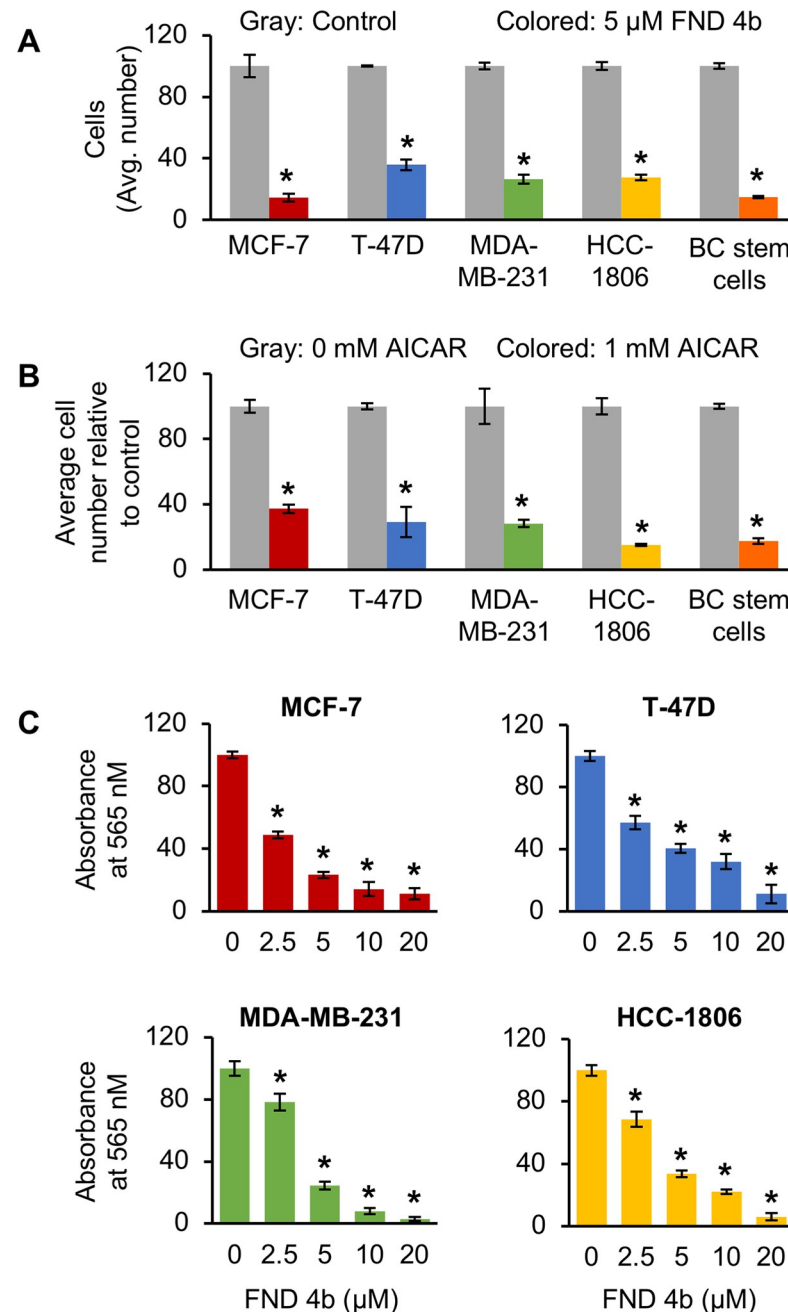
AMPK $\alpha$  has been implicated as a tumor suppressor in breast cancer [17–26]. Since FND-4b activated AMPK $\alpha$ , we measured its effects on the growth of breast cancer cells and compared with AICAR—a known AMPK activator. Cell counting assays showed that treatment at 5  $\mu$ M FND-4b for 72 h resulted in significant growth inhibition of MCF-7, T-47D, MDA-MB-231, HCC-1806, and breast cancer stem cells (Fig 3A). Similar decreases occurred in all breast cancer subtypes with 5  $\mu$ M treatment. Treatment at a much higher concentration of AICAR (1 mM) for 72 h yielded growth reductions similar to FND-4b among all subtypes (Fig 3B). The most striking differences were that MCF-7 cells were more sensitive to FND-4b while HCC-1806 cells were more responsive to AICAR. Only small differences were noted among T-47D, MDA-MB-231, and breast cancer stem cells. In addition, SRB growth assays indicated that treatment at various FND-4b concentrations (2.5, 5, 10, and 20  $\mu$ M) for 72 h yielded significant dose-dependent decreases in proliferation of MCF-7, T-47D, MDA-MB-231, and HCC-1806 cells (Fig 3C). ER+BC cells were more sensitive than TNBC cells to FND-4b at 2.5  $\mu$ M, but the reductions were similar at higher concentrations. Similar growth inhibition at the 5  $\mu$ M dosage between ER+BC and TNBC is consistent with the results from the cell counting assays. Taken together, these results illustrate that activation of AMPK $\alpha$  with FND-4b resulted in dose-dependent decreases in growth in ER+BC and TNBC.

## FND-4b increased apoptosis in ER+BC and TNBC

While FND-4b's major effect is on cell growth, its treatment also increased apoptosis in colon cancer [27]. Therefore, we investigated apoptosis induction in breast cancer cells. As previously mentioned, treatment with FND-4b resulted in increased levels of cleaved PARP, a marker of apoptosis, in MDA-MB-231, HCC-1806, and MCF-7 cells (see Fig 2). We also measured apoptosis with ELISA cell death assays that are more sensitive than western blotting assays. Significant increases in apoptosis were found in MCF-7 and T-47D cells—with MCF-7 cells being more sensitive (Fig 4A). Apoptosis was significantly increased in MDA-MB-231 cells with treatment at 10  $\mu$ M FND-4b, but apoptosis in HCC-1806 cells was not increased (Fig 4A). Finally, to determine whether higher baseline pAMPK levels affect apoptosis in these cells, we compared the basal rates of apoptosis among the four cell lines used in the ELISA assays. HCC-1806 cells, which had the lowest pAMPK levels at baseline (Fig 1), had a much higher basal apoptotic rate than the other three cell lines (Fig 4B). This indicates that higher baseline levels of pAMPK do not increase the basal apoptotic rate. The elevated basal level of apoptosis in HCC-1806 cells is likely the reason why FND-4b was unable to induce apoptosis in this cell line. FND-4b's ability to suppress growth of HCC-1806 cells resulted in fewer cells in the treated wells compared to the control wells. As a result, there were less cells that could have undergone apoptosis—resulting in lower readings in the treated wells.

## Discussion

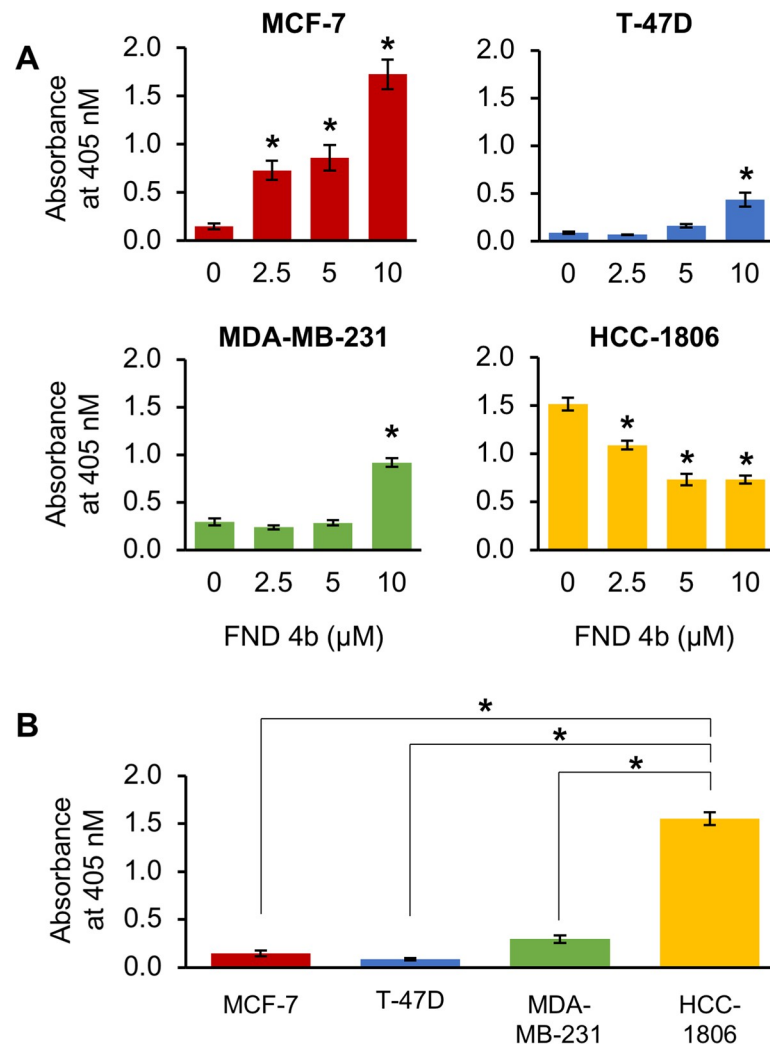
The connection between AMPK activation and the inhibition of cancer cell growth prompted our interest in targeting AMPK and its downstream signaling pathways. AMPK activators such as AICAR and 2-DG have limited utility for patient care due to their high dose requirements [12]. We focused on novel small-molecule agents that activated AMPK at low concentrations and that stood a greater chance than these well-known AMPK activators of progressing toward the clinic. In this project, we examined the effects of the AMPK activator FND-4b to determine its effects on ER+BC, TNBC, and breast cancer stem cells. FND-4b previously suppressed the growth of colorectal cancer cells and stem cells through activation of



**Fig 3. AMPK $\alpha$  activation with FND-4b decreased proliferation of breast cancer cells with similar efficacy to AICAR.** (A) Equal numbers of MCF-7, T-47D, MDA-MB-231, HCC-1806, or breast cancer stem cells were grown in medium containing 0 or 5  $\mu$ M FND-4b for 72 h followed by cell counting. (B) The same procedure described in (A) was followed with the exception of growing cells in medium containing 0 or 1 mM AICAR instead. (C) MCF-7, T-47D, MDA-MB-231, and HCC-1806 cells were grown in medium containing different concentrations of FND-4b (0, 2.5, 5, 10, and 20  $\mu$ M) for 72 h before SRB growth assays were performed. Data are presented as mean  $\pm$  SD from experiments performed in triplicate (cell counting) or sextuplicate (SRB assays) and are representative of three independent experiments. \* indicates p-value < 0.01.

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AMPK in the low micromolar range without affecting signaling through the Akt or ERK pathways [27, 28]. We found that treatment with FND-4b resulted in dose-dependent increases in AMPK activation in both breast cancer subtypes and in the stem cells.



**Fig 4. AMPK $\alpha$  activation with FND-4b increased apoptosis of breast cancer cells.** (A) Equal numbers of MCF-7, T-47D, MDA-MB-231, or HCC-1806 cells were cultured in medium containing different concentrations of FND-4b (0, 2.5, 5, and 10  $\mu$ M) for 72 h before ELISA cell death assays were performed. (B) The basal apoptotic rates of MCF-7, T-47D, MDA-MB-231, and HCC-1806 cells were compared using the control data from the ELISA cell death assays in (A). Data are presented as mean  $\pm$  SD from an experiment performed in triplicate and are representative of three independent experiments. \* indicates p-value < 0.001.

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Other investigators have also focused on discovering novel AMPK activators or repurposing current drugs that activate AMPK for breast cancer therapy. OSU-53 and RL-71 activated AMPK and exerted anti-tumor effects in TNBC at low micromolar doses [17, 19]. In addition, demethoxycurcumin (20  $\mu$ M) resulted in AMPK activation and large decreases in TNBC and ER+BC cell proliferation [24]. Finally, treatment of TNBC with the anti-depressant fluoxetine (0.5  $\mu$ M) caused AMPK activation and substantial reductions in cellular viability [25]. Taken together, these studies and ours suggest the merit in targeting the AMPK signaling pathway for the treatment of breast cancer.

We found that low micromolar concentrations of FND-4b substantially reduced the growth of TNBC. The decreases in proliferation that were induced by 5  $\mu$ M FND-4b were similar to those caused by a much higher concentration of AICAR (1 mM). Although HCC-1806 cells were more responsive to AICAR in cell counting assays, slightly higher concentrations of

FND-4b (10 or 20  $\mu$ M) should suppress HCC-1806 growth more than 1 mM AICAR. The substantial growth reductions that we found are particularly important because of the inherent aggressiveness of TNBC that has higher rates of recurrence and metastasis than other breast cancer subtypes [3]. The seriousness of TNBC is further amplified by the fact that most breast cancer deaths result from metastatic lesions [2]. The difficulty in developing treatments for TNBC is due to the lack of the estrogen receptor and HER2 amplification. Drugs that target these proteins, such as trastuzumab and tamoxifen, are ineffective in TNBC. Instead, patients with TNBC typically receive a drug cocktail that damages normal cells in addition to the tumor and leads to significant side effects. Current efforts are focused on developing drugs that specifically target proteins or pathways that are exclusively altered in TNBC. Since expression of AMPK and pAMPK is lower in breast cancer than normal tissue, this signaling pathway attracted our attention as an option for targeted therapy [30–32].

AMPK activation resulted in substantial decreases in cell proliferation in all breast cancer subtypes that were tested. These reductions in growth were due to AMPK's ability to regulate the cell cycle both directly and indirectly. Directly, AMPK activation can attenuate levels of cyclin D1, an important protein that controls cell cycle arrest during the G1 phase [33]. Prior work in ovarian cancer has suggested that AMPK activation causes degradation of cyclin D1 through a pathway involving glycogen synthase kinase 3 $\beta$  [33]. Once cyclin D1 is degraded, cells are prevented from progressing past the G1 phase [33]. We found that FND-4b-induced AMPK activation resulted in substantial decreases in cyclin D1 expression, resulting in cell cycle arrest. Indirectly, AMPK activation can affect the cell cycle through effects on mTOR and cellular metabolism. AMPK downregulates flux through the mTOR pathway, which can control the cell cycle through its downstream effectors S6 kinase 1 (S6K1) and eukaryotic translation initiation factor 4E-binding protein 1 [34]. In our study, we measured mTOR activity by blotting for levels of phosphorylated ribosomal protein S6, which is downstream from S6K1. We found decreases in S6 phosphorylation with FND-4b-induced AMPK activation, indicating less mTOR flux and cell cycle progression. Additionally, AMPK affects cell metabolism by phosphorylating and inhibiting ACC, which is the rate-limiting step in fatty acid synthesis. As a result, *de novo* lipogenesis is inhibited. Fatty acids are required for progression through the cell cycle—notably, during the G<sub>1</sub>-S and G<sub>2</sub>-M phases—and in their absence, cells will be unable to complete mitosis [16]. Instead they will be arrested at the G<sub>2</sub>-M checkpoint [16]. We showed that FND-4b-induced AMPK activation led to increased ACC phosphorylation, signifying less fatty acid synthesis and flux through the cell cycle.

In addition to inducing cell cycle arrest, AMPK can also act as a tumor suppressor by causing apoptosis [32]. We found that FND-4b causes apoptosis in a dose-dependent manner in ER+BC cells with ELISA cell death assays. We also showed clear increases in levels of cleaved PARP—an apoptotic indicator—in MCF-7 cells. TNBC cells were more resistant to apoptosis from FND-4b, but there was apoptosis in MDA-MB-231 cells with 10  $\mu$ M treatment. Additionally, we found increases in cleaved PARP in MDA-MB-231 and HCC-1806 cells with western blot. Taken together, these data indicate that ER+BC cells are more susceptible to FND-4b-induced apoptosis than TNBC. However, as suggested in previous work, the effects of FND-4b on cell cycle progression are more pronounced and consistent than on apoptosis [27].

## Conclusions

We have shown that the novel compound FND-4b can activate AMPK in ER+BC, TNBC, and breast cancer stem cells. In addition, treatment with this compound can dose-dependently decrease proliferation and increase apoptosis in breast cancer cells. The effects on cellular growth are mediated via decreased cell cycle flux—as evidenced by reductions in cyclin D1

levels—and suppression of fatty acid synthesis and mTOR signaling. With such profound effects on proliferation, further development of FND compounds could lead to their inclusion in TNBC treatment regimens.

## Supporting information

**S1 Fig. Data underlying the figures for the SRB growth assays.**  
(XLSX)

**S2 Fig. Data underlying the figures for the cell proliferation assays.**  
(XLSX)

**S3 Fig. Data underlying the figures for the ELISA cell death assays.**  
(XLSX)

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