

Sorafenib synergizes with metformin in NSCLC through AMPK pathway activation

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The multikinase inhibitor sorafenib is under clinical investigation for the treatment of many solid tumors, but in most cases, the molecular target responsible for the clinical effect is unknown. Furthermore, enhancing the effectiveness of sorafenib using combination strategies is a major clinical challenge. Here, we identify sorafenib as an activator of AMP-activated protein kinase (AMPK), in a manner that involves either upstream LKB1 or CAMKK2. We further show in a phase II clinical trial in *KRAS* mutant advanced non-small cell lung cancer (NSCLC) with single agent sorafenib an improved disease control rate in patients using the antidiabetic drug metformin. Consistent with this, sorafenib and metformin act synergistically in inhibiting cellular proliferation in NSCLC *in vitro* and *in vivo*. A synergistic effect of both drugs is also seen on phosphorylation of the AMPK α activation site. Our results provide a rationale for the synergistic antiproliferative effects, given that AMPK inhibits downstream mTOR signaling. These data suggest that the combination of sorafenib with AMPK activators could have beneficial effects on tumor regression by AMPK pathway activation. The combination of metformin or other AMPK activators and sorafenib could be tested in prospective clinical trials.

Key words: AMP-activated protein kinase, metformin, non-small cell lung cancer, salicylate, sorafenib

Abbreviations: ACC: acetyl-CoA carboxylase; AMPK: AMP-activated protein kinase; DCR: disease control rate; HCC: hepatocellular carcinoma; NAC: *N*-acetyl-L-cysteine; NSCLC: non-small cell lung cancer; ORR: overall response rate; OS: overall survival; PFS: progression-free survival; PR: partial response; ROS: reactive oxygen species; SD: stable disease

Additional Supporting Information may be found in the online version of this article.

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Introduction

AMP-activated protein kinase (AMPK) signaling has increasingly attracted interest in carcinogenesis because AMPK acts as a metabolic checkpoint coordinating cellular growth. AMPK acts through inhibition of cell cycle progression and repression of the mTOR pathway, a pathway that is frequently activated in cancer. AMPK is activated in response to AMP binding or by phosphorylation at threonine-172 (Thr172) on the alpha-subunit by the tumor suppressor protein kinase *LKB1* (also called *STK11*) or the calcium activated kinase *CAMKK2*.¹ *LKB1* is of particular interest because mutations in the *LKB1* gene are responsible for Peutz–Jeghers syndrome, a rare autosomal dominant disorder with a strong tendency of developing cancer.² In addition, cumulative evidence suggests that somatic mutations or inactivation of the *LKB1* gene is involved in lung and cervical cancers.³ Gill *et al.* found in non-small cell lung cancers (NSCLCs) a generally low rate of somatic mutations (11%) in the *LKB1* gene, while they observed that either loss of heterozygosity or homozygous deletion of the *LKB1* gene occurred in nearly 90% of the NSCLCs tested.⁴

The oral antidiabetic drug metformin belongs to the biguanide class and is the first-line drug of choice in the treatment of type II diabetes. Several epidemiological and case-controlled studies found diabetics using metformin have a up to 30% lower lifetime cancer risk in comparison to

What's new?

As cells acquire cancerous phenotypes, they experience significant changes in metabolism. A key regulator of those changes is AMP-activated protein kinase (AMPK), which serves a complex role in cancer. Here, the multikinase inhibitor sorafenib was found as an activator of AMPK, via involvement of LKB1 and CAMKK2. When sorafenib was combined with the AMPK activator metformin, synergistic growth inhibition was observed in NSCLC cells *in vitro* and *in vivo*. The combination was found to improve disease control rate in a phase II clinical trial of patients with KRAS mutant advanced non-small cell lung cancer (NSCLC).

those using other antidiabetic medications.^{5–7} In line with this reduced cancer risk, metformin is activating AMPK and exhibits an antiproliferative effect on several cancer cell lines.⁸ Furthermore, metformin and the allosteric AMPK activator A-769662 both delay spontaneous tumor development in *Pten*^{+/-} mice,⁹ but it has been unclear whether the reduced cancer incidence in diabetics is also explained by AMPK activity. Other mechanisms can be proposed to explain the effects of metformin on cancer, like the insulin-sensitizing and antihyperglycemic effects.^{7,10} The activation of AMPK by metformin can be *direct* through an increase in AMP/ATP ratio or can be *indirect* through its activity on upstream kinases.⁷

Mechanistic studies have shown that AMPK plays an important role as well in the mechanism of action of thiazolidinedione's¹¹ and statins.¹² More recently, salicylate-the active metabolite of aspirin-was also found to act as an AMPK activator.¹³ Aspirin's effect on cancer has been widely studied, particularly its effect on colorectal cancer incidence and mortality. Strikingly, it was reported that only in patients with mutated-*PIK3CA* colorectal cancer the regular use of aspirin after diagnosis was associated with longer survival, and not among patients with wild-type *PIK3CA* colorectal cancer.¹⁴

Metformin might be an attractive and safe anticancer drug in monotherapy or in combination with chemotherapeutic or targeted agents. To date, no randomized controlled trials of metformin as a cancer therapy have been reported, but (pre-) clinical evidence suggests that metformin may enhance chemotherapy response.^{15–19} However, it is also reported that metformin can drive angiogenesis and accelerate the *in vivo* growth of *BRAF*^{V600E}-driven melanoma by upregulating *VEGFA* mRNA and protein levels.²⁰ Multiple combinations of metformin and targeted agents are currently under clinical investigation (<http://www.clinicaltrials.gov/>).

Here, we report on *post hoc* analysis of a phase II clinical trial with the multikinase inhibitor sorafenib in *KRAS* mutant NSCLC²¹ that patients receiving metformin during sorafenib treatment showed improved disease control rate (DCR). We observed synergistic growth inhibition of NSCLC cells *in vitro* and *in vivo* with the combination of sorafenib and metformin and describe synergistic AMPK activation and downstream mTOR pathway inhibition as the mechanism explaining the effects of the combination. In our study, we

identify sorafenib as an activator of AMPK, in a manner that involved either upstream LKB1 or CAMKK2.

Material and Methods**Phase II clinical trial of sorafenib in KRAS mutant advanced NSCLC**

Previously, Dingemans *et al.* reported the activity of sorafenib monotherapy in a single arm phase II trial in patients with locally advanced or metastatic nonsquamous NSCLC harboring a *KRAS* mutation (Dutch trial register NL30000.029.09).²¹ We used their study to perform a *post hoc* analysis on the group of patients receiving metformin for their type II diabetes. The primary endpoint of their study was DCR, defined as no progression after 6 weeks of start treatment (RECIST 1.0 criteria). Secondary endpoints were overall response rate, duration of response, progression free survival (PFS) and overall survival.

Reagents

Sorafenib (cat. no. S1040) was purchased from Selleck Chemicals. Metformin hydrochloride (cat. no. PHR1084), sodium salicylate (cat. no. S2679), Hydrogen Peroxide solution (cat. no. 216763) and *N*-acetyl-L-cysteine (cat. no. A9165) were purchased from Sigma Aldrich. STO-609 (cat. no. sc-202820) was obtained from Santa Cruz Biotechnology.

Cell Culture and Viral Transduction

A549, H358, H460, H522 and H838 cells were purchased from American type culture collection (ATCC) and Huh-7 cells from the Japanese collection of research bioresources (JCRB) cell bank. An overview of the *KRAS* and *LKB1* status of these cell lines is shown in Supporting Information Table 3. The cells were cultured in DMEM (Huh-7) or RPMI (other cell lines) supplemented with 8% heat-inactivated fetal calf serum and 1% penicillin/streptomycin at 5% CO₂.

HEK293T cells were used as producers of lentiviral supernatants as described (<http://www.broadinstitute.org/rnai/public/resources/protocols>). The calcium phosphate method was used for the transfection of 293T cells. Infected cells were selected for successful lentiviral integration using 2 µg/ml of puromycin.

Plasmids

All lentiviral shRNA vectors were retrieved from the arrayed TRC human genome-wide shRNA collection. The following RNAi target sequences were used: shCAMKK2#1, CGA

GCGGATCATGTGTTTACA; shCAMKK2#2, CCGTTTCTA CTTCCAGGATCT; shLKB1, GATCCTCAAGAAGAAGAA GTT. Control infections were performed with the empty pLKO.1 vector.

Colony formation assays

Cells were seeded in 6-well plates ($5-10 \times 10^3$ cells per well) and cultured both in the absence and presence of drugs as indicated for 10 days. Cells were washed with phosphate buffered saline (PBS), fixed with 4% formaldehyde in PBS and stained with 0.1% crystal violet.

Protein lysate preparation and immunoblots

The biochemical responses of cells treated with drugs were analyzed by Western blot. Cells were seeded in six well plates in medium containing 8% fetal calf serum. After 12 hr, cells were treated with drugs for the indicated time without refreshing the medium. The lysates were collected using radioimmunoprecipitation assay (RIPA) buffer containing 150 mM NaCl, 50 mM Tris pH 8.0, 1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS supplemented with protease inhibitors (Complete, Roche) and Phosphatase Inhibitor Cocktails II and III (Sigma). All lysates were freshly prepared, normalized using bicinchoninic acid (BCA) protein assay (Thermo Scientific) and resolved by SDS gel electrophoresis and followed by western blotting. Primary antibodies against p-AMPK α Thr172 (40H9; #2535), AMPK α (#2532), LKB1 (26D10; #3050), p-ACC Ser79 (#3661), ACC (#3662), p-S6 RP Ser240/244 (#2215), p-S6 RP Ser235/236 (#2211), S6 RP (5G10; #2217) p-4E-BP1 Ser65 (174A9; #9456) and 4E-BP1 (#9452) were from Cell Signaling Technology and HSP 90 α/β (H-114; sc-7947) from Santa Cruz Biotechnology. Secondary antibody was obtained from Bio-Rad Laboratories.

Quantitative RT-PCR

The 7500 Fast Real-Time PCR System from Applied Biosystems was used to measure mRNA levels. mRNA expression levels were normalized to expression of GAPDH. The following primer sequences were used in the SYBR Green master mix (Roche): GAPDH_forward, 5'-AAGGTGAAGGTCGGAGTCAA-3'; GAPDH_reverse, AATGAAGGGGTCATTGATGG; VEGFA_forward, 5'-CCCACTGAGGAGTCCAACAT-3'; VEGFA_reverse, 5'-TTTCTTGCGCTTTTCGTTTTT-3'.

Mouse xenografts and *in vivo* drug study

All experimental procedures on animals were approved by the Animal Ethics Committee of the Netherlands Cancer Institute in accordance with the Dutch Act on Animal Experimentation. A549 cells (1.8×10^6 cells per mouse) were injected subcutaneously into the right posterior flanks of 7-week-old immunodeficient Balb/c female nude mice (5–8 mice per group) (Charles River). Tumor formation was monitored every other day, and tumor volume based on caliper measurements was calculated by the modified ellipsoidal formula: tumor volume = $0.5 \times \text{length} \times \text{width}^2$. When tumors reached a vol-

ume of approximately 50 mm³, mice were randomly assigned to treatment with vehicle [PEG400 in sterile PBS (1:1) by daily gavage], sorafenib (30 mg/kg of body weight by daily gavage), metformin (400 mg/kg/day dissolved in the drinking water, assuming an average water consumption per day per mouse as was calculated before the start of the study) or to the drug combination (sorafenib plus metformin), in which each compound was administered at the same dose and schedule as single agents. Sorafenib for *in vivo* study was dissolved in dimethyl sulfoxide (DMSO), stored in aliquots at -80°C and diluted in vehicle before administration.

Immunohistochemistry

Tumor sections from FFPE A549 xenograft tumors were stained for p-4E-BP1 [Thr37/46 (#2855), dilution 1:5000; Cell Signaling]. Detection was performed using CSA II signal amplification system (Dako). Sections were scored by two pathologists (H.M.H. and S.M.W.) using the semiquantitative *H*-score that takes into consideration the staining intensity (0–3+) in conjunction with the percentage of viable tumor cells staining positively. *H*-score = (% at 0) * 0 + (% at 1+) * 1 + (% at 2+) * 2 + (% at 3+) * 3. Thus, this score produces a continuous variable that ranges from 0 to 300.

Tumor vessel density was analyzed in FFPE A549 xenograft tumors. Sections were stained for CD31 [Anti-CD31 (#ab28364), dilution 1:50; Abcam]. Detection was performed using the EnVisionTM+ system (Dako). Two pathologists (H.M.H. and S.M.W.) counted the number of CD31-positive vessels in 10 randomly clockwise selected fields (200 \times magnification) containing viable tumor. Vessel density was calculated as the sum of CD31-positive vessels in the 10 fields.

Statistical analysis

The differences in response rate in the Phase II clinical trial between patients not using metformin and patients using metformin were tested using a Chi-square test with three categories (two-tailed *p*-value). Differences in tumor volume, tumor growth rate, phospho-4E-BP1 immunostaining and number of CD31-positive vessels between treatment groups from the xenograft study were tested for significance using an unpaired T-test (two-tailed *p*-value) in GraphPad Prism 6 software. Combination treatment was assessed using a linear regression of tumor growth rate on treatment groups including an interaction term using SPSS software. *p*-Values < 0.05 were considered as statistically significant.

Results

Improved DCR in *KRAS* mutant advanced NSCLC patients receiving metformin during sorafenib treatment

In the context of a phase II clinical trial (see Methods section), a total of 57 advanced *KRAS* mutant heavily pretreated NSCLC patients were treated with monotherapy sorafenib.²¹ Five patients were using metformin during the treatment because of type II diabetes. No other patients with diabetes participated in the study. Basic characteristics of the patients

Table 1. Responses according to the RECIST criteria after six weeks of sorafenib treatment in a phase II trial in patients with locally advanced or metastatic non-squamous NSCLC harboring a *KRAS* mutation ($n = 57$)

	Metformin		Total	<i>p</i> -value
	No	Yes		
Partial response	3	2	5	0.01
Stable disease	22	3	25	
Progressive disease	27	0	27	
Total	52	5	57	

in the metformin group and the nonmetformin group are given in Supporting Information Table 1.

The overall DCR was 52.6% and thereby sorafenib was found active. In the five patients using the combination of sorafenib and metformin the DCR was 100%; two patients with partial response (PR) and three patients with stable disease (SD). In the 52 remaining patients, three patients had a PR, 22 patients had SD and 27 patients had progressive disease (Table 1; $p = 0.01$). The two patients with PR in the metformin group had duration of response of 10 and 13 months. The three patients with PR in the nonmetformin group had duration of response of 1, 5 and 5 months. Median PFS was 2.2 months (95% CI 1.1–3.3) in the nonmetformin group and 2.8 months (95% CI 2.4–3.1) in the metformin group ($p = 0.06$). It is interesting to note that the two patients with PR in the metformin group had the longest PFS of all patients in our study. Median OS was 4.8 months (95% CI 1.5–8.2) in the nonmetformin group and 9.0 months (95% CI 0.1–17.9) in the metformin group ($p = 0.13$). The two patients with PR in the metformin group also had one of the longest survivals; one patient was censored at 12 months and the other patient had a survival of 14 months.

Synergistic effect of sorafenib in combination with metformin or other AMPK activators in NSCLC cell lines

To study a potential interaction between sorafenib and metformin, we tested this drug combination in a 10-day colony formation assay using the *KRAS* mutant NSCLC cell lines A549 and H460. As shown in Figures 1a and 1b, combination treatment of sorafenib and metformin caused a synergistic inhibition in proliferation in both A549 and H460 cells. To test whether AMPK activation by metformin underlies the synergistic inhibition of proliferation we treated cells with sorafenib in combination with the allosteric AMPK activator A-769662 or the AMPK activator salicylate. Figures 1c–1f show that these compounds also act synergistically with sorafenib in inhibiting proliferation of A549 and H460 cells. Quantifications of the colony formation assays in Figure 1 are shown in Supporting Information Table 4.

Besides a *KRAS* mutation, A549 and H460 cells also harbor an inactivating mutation in the *LKB1* gene. We therefore tested whether the combination of sorafenib and an AMPK

activator would also synergistically inhibit the proliferation of *LKB1* wild-type NSCLC cells. Colony formation assays of sorafenib in combination with salicylate in *LKB1* wild-type *KRAS* mutant H358 cells, *LKB1* mutant *KRAS* wild-type H838 cells and double wild-type H522 NSCLC cells exhibited that the effect of the combination is independent of *LKB1* or *KRAS* mutation status (Supporting Information Fig. 1).

Synergistic antiproliferative effect through AMPK activation and downstream mTOR inhibition

It has been shown that the antiproliferative effect of metformin is mediated by AMPK activation.^{8,19} We have therefore measured phosphorylation of AMPK α at its activation site Thr172 after treatment of cells with sorafenib, metformin or the combination thereof. Figures 2a and 2b show an unexpected activation of AMPK α , as judged by Thr172 phosphorylation, by sorafenib treatment alone. Importantly, AMPK activation was increased significantly when sorafenib was combined with metformin, independent of *LKB1* or *KRAS* mutation status (Figs. 2a and 2b). Increased AMPK activation was also observed when cells were treated with sorafenib in combination with the AMPK activators salicylate (Fig. 2c) or A-769662 (Supporting Information Fig. 2a). The antiproliferative effect of AMPK activation is mediated, at least in part, by suppression of mTOR signaling. Indeed, combination treatment showed a synergistic effect on inhibition of phosphorylation of the downstream mTOR targets 4E-BP1 and ribosomal protein S6 (Figs. 3a and 3b). Beside mTOR, AMPK has many more substrates, one of them being acetyl-CoA carboxylase (ACC).¹ ACC is the key enzyme in the biosynthesis and oxidation of fatty acids. Phosphorylation by AMPK at Ser79 inhibits the enzymatic activity of ACC. Treatment of cells with either sorafenib; metformin or salicylate indeed resulted in increased phosphorylation of ACC at Ser79 (Figs. 3a and 3b). Phosphorylation was increased most when cells were treated with the combination of sorafenib and metformin or salicylate (Figs. 3a and 3b).

Sorafenib activates AMPK in both an LKB1-dependent and LKB1-independent, but CAMKK2-dependent manner

Activation of AMPK can be brought about through two independent upstream signaling routes: one involving *LKB1* and a second Ca²⁺-dependent signaling route involving CAMKK2.¹ To gain more insight into how sorafenib activates AMPK, we used a number of genetic and chemical inhibitors of these signaling routes. Figure 4a shows that treatment with sorafenib leads to activation of AMPK in both *LKB1* wild-type and mutant NSCLC cells in a concentration dependent manner. To extend this finding, we asked whether sorafenib also activates AMPK in hepatocellular carcinoma (HCC) cells, because sorafenib is already approved for clinical use in HCC. Figure 4e shows that treatment of *LKB1* wild-type Huh-7 HCC cells with sorafenib also activates AMPK. Furthermore, we show that treatment of cells with fluorosorafenib (known as regorafenib) activates AMPK to a similar

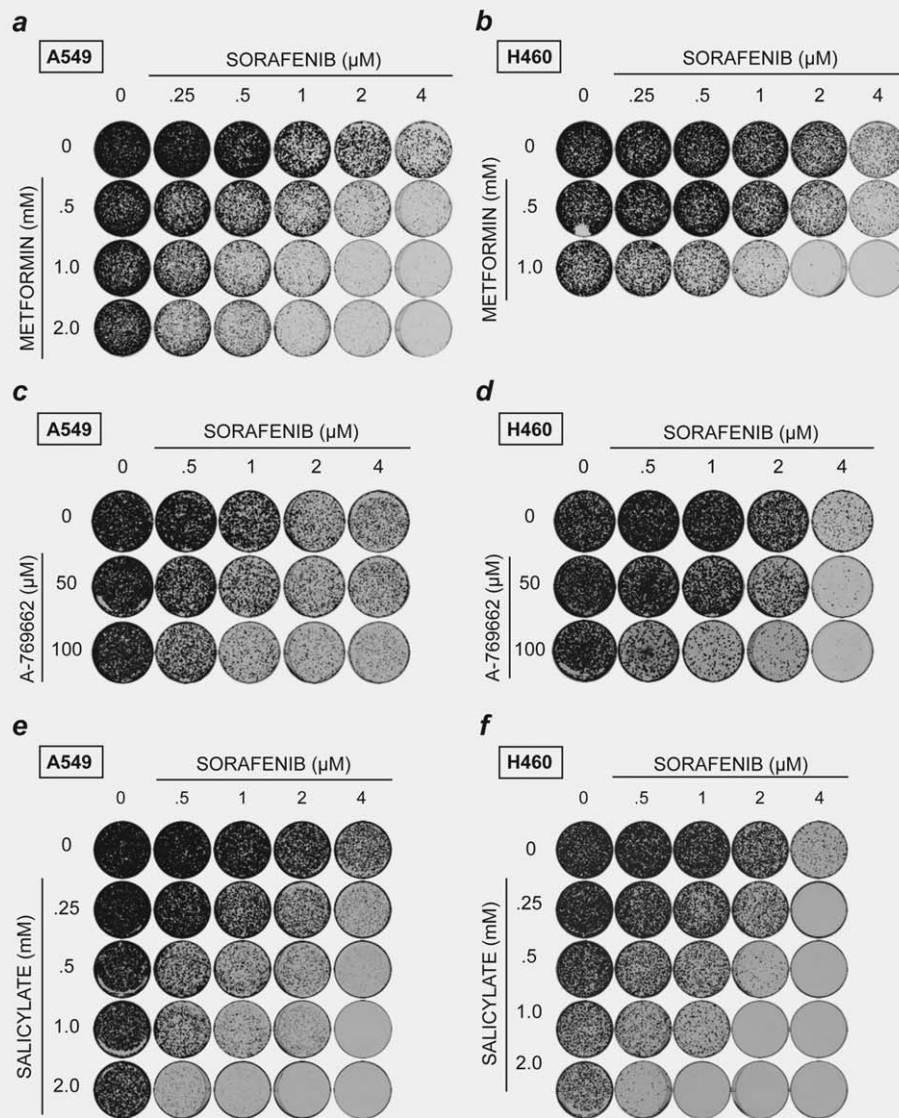


Figure 1. Sorafenib synergizes with metformin and other AMPK activators in NSCLC cell lines. *a–f*, Colony formation assay of A549 (*a, c, e*) and H460 (*b, d, f*) NSCLC cells with increasing concentrations of sorafenib (0–4 μ M) in the absence or presence of increasing concentrations of metformin (0–2 mM) (*a, b*); A-769662 (0–100 μ M) (*c, d*); or salicylate (0–2 mM) (*e, f*). Cells were grown in six-well plates and refreshed every 3 days. The cells were fixed, stained and photographed after 10 days.

extent as sorafenib (Supporting Information Fig. 2*b*). Sorafenib is unique in activation of AMPK as other multikinase inhibitors, such as sunitinib (Sutent) or tivozanib, failed to increase p-AMPK α levels in cells (data not shown).

Next, we examined whether in *LKB1* mutant A549 and H460 cells the *LKB1*-independent route is mediated by the protein kinase *CAMKK2*. As shown in Figures 4*b* and 4*c*, inactivation of *CAMKK2* in these cells, either by shRNA knockdown or by chemical inhibition, blocked AMPK activation by sorafenib. In contrast, knockdown of *CAMKK2* or chemical *CAMKK* inhibition failed to block the activation of AMPK by sorafenib in *LKB1* wild-type H358 cells (Figs. 4*b*

and 4*c*). Consistent with this, we found that *CAMKK2* knockdown in *LKB1* wild-type Huh-7 HCC cells is also not sufficient to inhibit the activation of AMPK by sorafenib (Fig. 4*e*). We next examined the effect of *LKB1* knockdown in *LKB1* wild-type cells on AMPK activation. As shown in Figure 4*d*, *LKB1* knockdown reduced the basal AMPK α phosphorylation and thereby AMPK activity in H358 cells, although sorafenib could still activate AMPK in these cells. It is possible that this residual AMPK activation is mediated by *CAMKK2*. Consistent with this, we found that treatment with the *CAMKK* inhibitor STO-609 completely blocked the AMPK activation by sorafenib in these H358 *LKB1* knockdown cells (Fig. 4*d*).

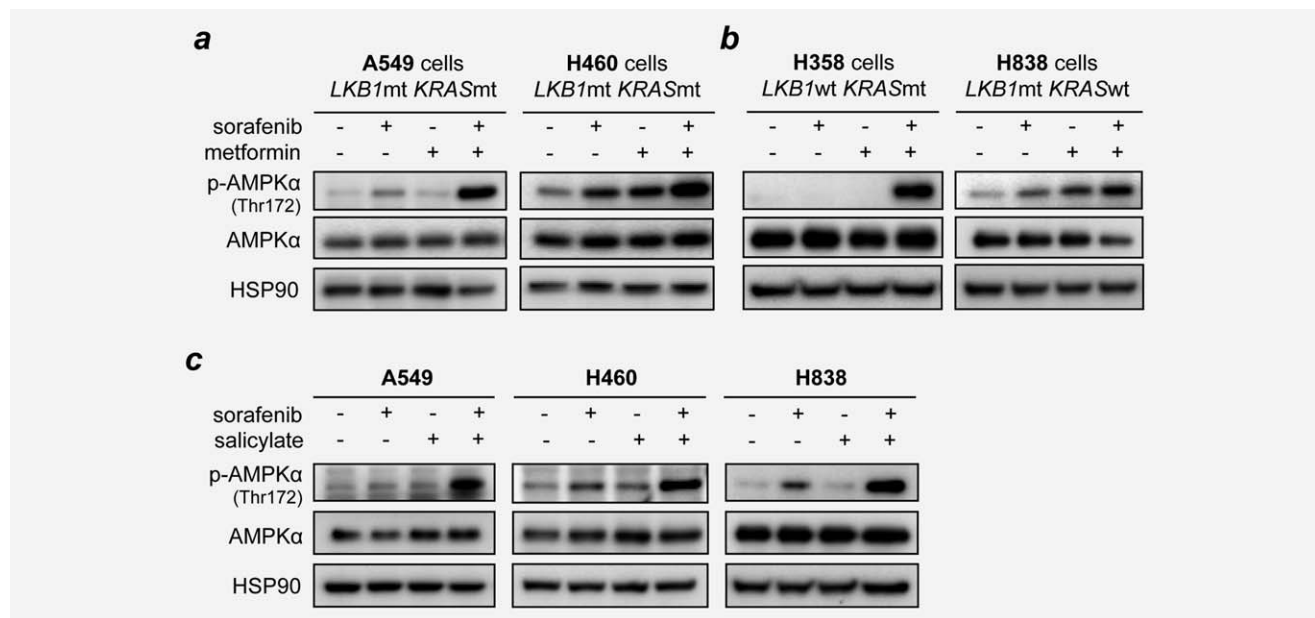


Figure 2. Sorafenib in combination with metformin or the AMPK activator salicylate enhances AMPK activation. *a, b*, AMPK activation with the combination of sorafenib and metformin in *LKB1* mutant *KRAS* mutant (A549 and H460) NSCLC cells (*a*), *LKB1* wild-type *KRAS* mutant (H358) (*b*, left panel) or *LKB1* mutant *KRAS* wild-type (H838) NSCLC cells (*b*, right panel). Cells were treated for 48 hr with sorafenib (1–3 μ M), metformin (1–1.5 mM) or the combination of sorafenib and metformin with the same concentrations as were used for the individual treatments. *c*, AMPK activation with the combination of sorafenib and salicylate in *LKB1* mutant *KRAS* mutant (A549 and H460) or *LKB1* mutant *KRAS* wild-type (H838) NSCLC cells. Cells were treated for 48 hr with sorafenib (1–3 μ M), salicylate (1–1.5 mM) or the combination of sorafenib and salicylate with the same concentrations as were used for the individual treatments. Cell lysates were harvested for western blot analysis and probed with the indicated antibodies.

Together, these data indicate that sorafenib activates AMPK through two redundant signaling routes that involve *LKB1* and *CAMKK2*, respectively. Only when both kinases are blocked, AMPK activation by sorafenib is compromised.

To further address the mechanism underlying the AMPK activation by sorafenib, we tested whether the *LKB1*-independent AMPK activation mediated by *CAMKK2* is calcium-dependent. We therefore, cotreated *LKB1* mutant A549 and H460 cells with sorafenib and increasing concentrations of the cell permeable calcium chelator BAPTA-AM. AMPK activation by sorafenib was reduced in the presence of BAPTA-AM in *LKB1* mutant A549 and H460 cells (Supporting Information Fig. 2c), indicating that the *CAMKK2*-mediated AMPK activation by sorafenib is dependent on cytosolic calcium release.

It has been reported that sorafenib can rapidly provoke the production of reactive oxygen species (ROS) and induce apoptotic death of cells through a mitochondria-dependent oxidative stress mechanism.²² The significance of this finding was further highlighted by Coriat *et al.*,²³ who found that the effectiveness of sorafenib in HCC cell lines *in vitro* is mediated by ROS production. More interestingly, they showed using sera from HCC patients that in response to sorafenib HCC cancer cells produce massive amounts of ROS, which in turn induce tumor cell death. Their data indicate that no or weak ROS production predicts a lack of sorafenib effectiveness.

We accordingly tested whether activation of AMPK in our cells was mediated via ROS production, as reported previously.^{24,25} We found that 1-hr treatment with 0.4 mM hydrogen peroxide (H_2O_2 , which produces ROS) resulted in highly increased p-AMPK α levels in *LKB1* wild-type H358 cells, whereas in *LKB1* mutant H460 cells the increase in p-AMPK α after treatment with H_2O_2 was less abundant (Fig. 4f). AMPK activation by H_2O_2 could be blocked by cotreatment of H358 cells with the ROS scavenger *N*-acetyl-L-cysteine (Fig. 4h). We also found that AMPK activation by H_2O_2 in *LKB1* mutant H460 cells was mediated via *CAMKK2*, as cotreatment of cells with H_2O_2 and the *CAMKK* inhibitor STO-609 effectively blocked AMPK activation (Fig. 4f). In contrast, we still observed a high amount of phosphorylated AMPK α when we treated *LKB1* wild-type H358 cells with a combination of H_2O_2 and STO-609 (Fig. 4f). Consistent with the notion that *LKB1* and *CAMKK2* can independently activate AMPK, we found that p-AMPK α levels in H358 *LKB1* knockdown cells were greatly reduced when cells were treated with the combination of H_2O_2 and STO-609 (Fig. 4g). Together, these data indicate that the activation of AMPK by ROS, like the activation by sorafenib, is dependent on either *LKB1* or *CAMKK2*. Our proposed model how sorafenib treatment leads to AMPK activation in *LKB1* wild-type and *LKB1* mutant cells is shown in Supporting Information Figure 2d.

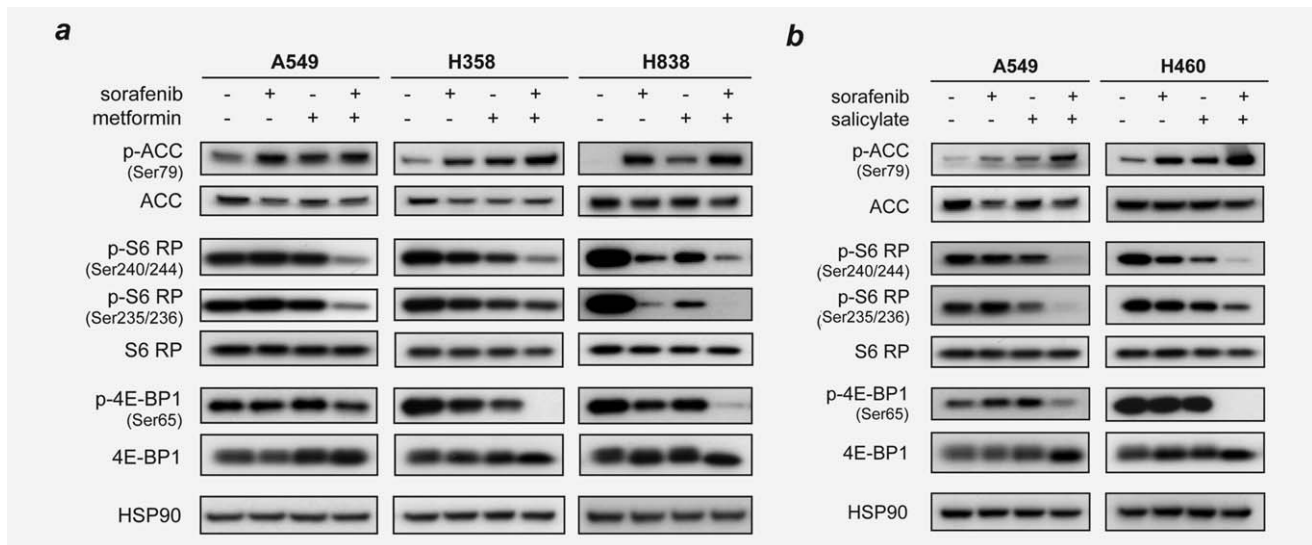


Figure 3. Sorafenib in combination with metformin or the AMPK activator salicylate represses the mTOR targets p-S6 and p-4E-BP1. *a*, Western blot analysis with the combination of sorafenib and metformin in *LKB1* mutant *KRAS* mutant (A549), *LKB1* wild-type *KRAS* mutant (H358) or *LKB1* mutant *KRAS* wild-type (H838) NSCLC cells. *b*, Western blot analysis with the combination of sorafenib and salicylate in *LKB1* mutant *KRAS* mutant NSCLC cells. Cells were treated for 48hrs with either sorafenib (1–3 μ M), metformin (1–1.5 mM) (*a*) or salicylate (1–1.5 mM) (*b*) alone or the combination of sorafenib with metformin/salicylate, with the same concentrations as were used for the individual treatments. Cell lysates were harvested for western blot analysis and probed with the indicated antibodies.

The effect of sorafenib in combination with metformin in a tumor xenograft model

To further validate the effectiveness of the combination of sorafenib and metformin for the treatment of NSCLC, we treated nude mice bearing A549 xenografts with vehicle, sorafenib (30 mg/kg/day), metformin (400 mg/kg/day) or the combination of sorafenib and metformin. Surprisingly, the tumor growth rate was higher for the metformin treated mice than for the vehicle treated mice, although this difference was not statistically significant (Supporting Information Table 2; $p = 0.097$). Mean tumor volume after 40 days treatment was significantly lower in the group treated with the combination compared to the group treated with sorafenib monotherapy (Fig. 5*b*; $p = 0.046$), with 6 of 8 mice in the combination group having a smaller tumor volume than the mice in the sorafenib group (Fig. 5*b*). Also, the mean tumor growth rate was significantly lower in the group treated with the combination compared to sorafenib monotherapy (Fig. 5*c*; $p = 0.023$). Based on a linear regression model, the mean tumor growth rate for the vehicle treated group was 7.2 mm³/day (Supporting Information Table 2). Separately, sorafenib and metformin lead to a nonsignificantly increased mean tumor growth rate (increased by 1 and 3.9 mm³/day over vehicle, respectively), whereas the combination of both treatments does result in a decrease of the mean tumor growth rate by 2.9 mm³/day compared to vehicle. The interaction coefficient (–7.8) was calculated by the difference between the expected mean growth rate of the combination (1 mm³/day plus 3.9 mm³/day) and the observed mean growth rate of the combination compared to vehicle

(–2.9 mm³/day). This interaction was statistically significant ($p = 0.026$; Supporting Information Table 2).

Tumor xenograft sections from the different treatment groups were immunostained for phospho-4E-BP1 and scored using the semiquantitative *H*-score (Fig. 5*d*). Tumors treated with the combination had a significant lower *H*-score compared to vehicle ($p = 0.048$) or sorafenib ($p = 0.022$), although the *H*-score was not significantly different from tumors treated with metformin. Figure 5*e* shows representative images of phospho-4E-BP1 stained tumor sections from vehicle and combination-treated tumors.

Discussion

We report here the ability of sorafenib to activate AMPK in NSCLC and HCC cells. This effect of sorafenib is greatly enhanced in combination with the known AMPK activator metformin, providing a rationale for synergism of these drugs to inhibit proliferation of NSCLC cells *in vitro* and *in vivo*.

The anticancer activity of sorafenib has been linked to its multikinase inhibitory action on several major signaling pathways. However, the role of additional pathways involved in the cytostatic and cytotoxic effects of sorafenib has obtained much attention in the last years. It has been reported that mitochondrial and endoplasmic reticulum stress induced by sorafenib is relevant for its effect on cell death.^{22,23,26} The mitochondria-dependent oxidative stress leads to the production of ROS and intracellular glutathione (an antioxidant) depletion. It was also shown that sorafenib can trigger cytosolic calcium mobilization and mitochondrial calcium overload.²² Rahmani *et al.* showed that the increased cytosolic

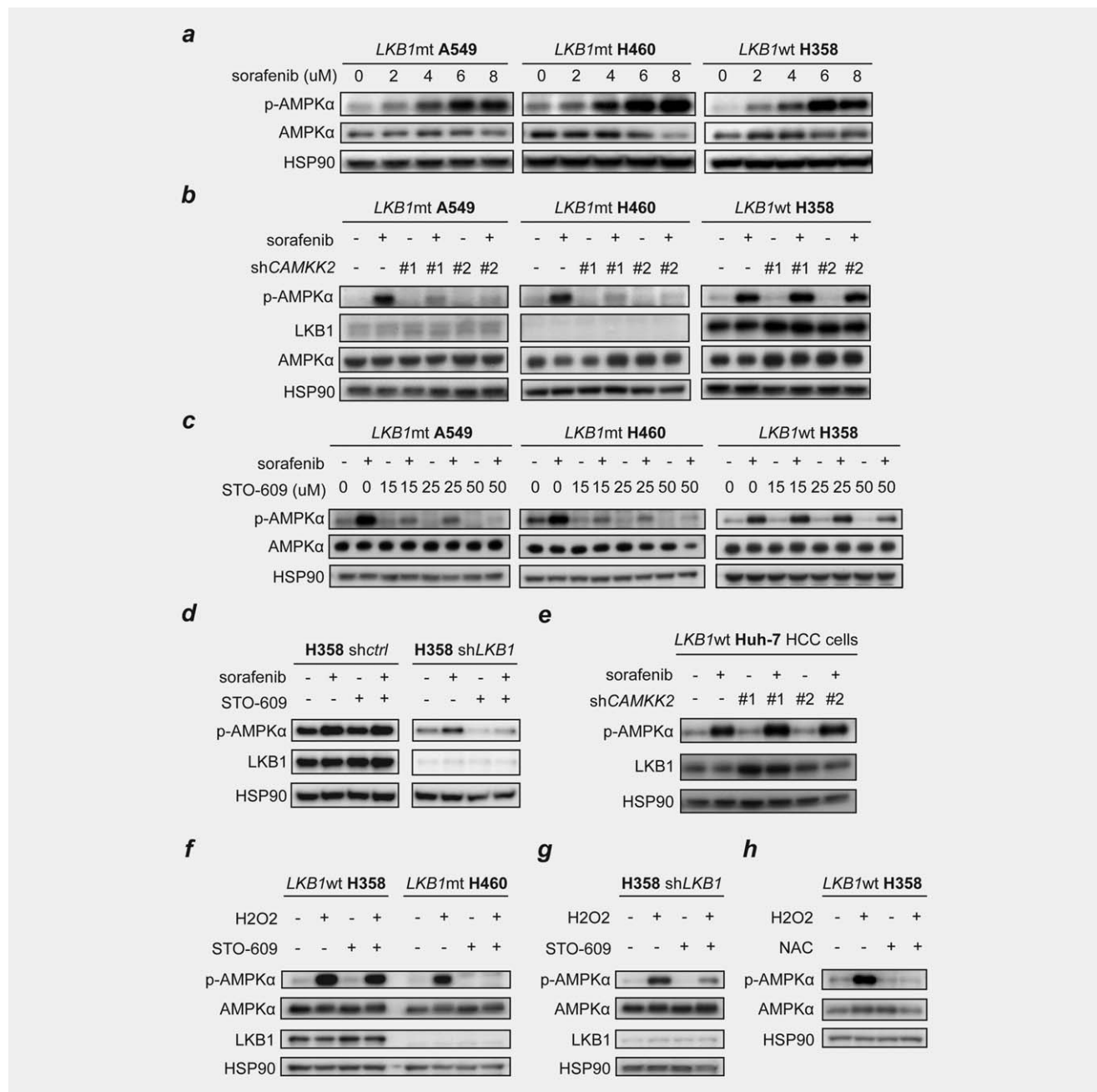


Figure 4. Activation of AMPK by sorafenib is dependent on the AMPK kinases *LKB1* and *CAMKK2*. *a*, Concentration dependent AMPK activation by sorafenib in *LKB1* mutant (A549 and H460) and *LKB1* wild-type (H358) NSCLC cells. Cells were treated for 6 hr with increasing concentrations of sorafenib (0–8 μM). *b* and *c*, AMPK activation by sorafenib is *CAMKK2*-dependent in *LKB1* mutant (A549 and H460) and *CAMKK2*-independent *LKB1* wild-type (H358) NSCLC cells. *b*, Cells expressing shctrl or sh*CAMKK2* were treated for 6 hr with 6 μM sorafenib. *c*, Cells were treated for 6 hr with increasing concentrations of the *CAMKK* inhibitor STO-609 (0–50 μM) in absence or presence of 6 μM sorafenib. *d*, AMPK activation by sorafenib is mainly *LKB1*-dependent in *LKB1* wild-type H358 cells. H358 cells expressing shctrl (left panel) or sh*LKB1* (right panel) were treated with the *CAMKK* inhibitor STO-609 (0–50 μM) in absence or presence of 6 μM sorafenib for 6 hr. The p-AMPK blot in *d* is relatively high exposed to highlight to differences in AMPK phosphorylation between shctrl cells and sh*LKB1* H358 cells. *e*, AMPK activation by sorafenib is also seen in *LKB1* wild-type hepatocellular carcinoma (HCC) Huh-7 cells, independent of *CAMKK2*. Huh-7 cells expressing shctrl or sh*CAMKK2* were treated for 6 hr with 6 μM sorafenib. *f*, AMPK activation by induced oxidative stress is mainly *LKB1*-dependent in *LKB1* wild-type (H358) and completely *CAMKK2*-dependent in *LKB1* mutant (H460) NSCLC cells. Cells were treated for 1-hr with 400 μM H₂O₂ in absence or presence of 50 μM of the *CAMKK* inhibitor STO-609. *g*, H358 cells expressing sh*LKB1* were treated for 1-hr with 400 μM H₂O₂ in absence or presence of 50 μM of the *CAMKK* inhibitor STO-609. Cells treated with the combination were pre-treated for 30 minutes with the *CAMKK* inhibitor before adding H₂O₂. *h*, AMPK activation by induced oxidative stress is blocked with the ROS-scavenger *N*-acetyl-L-cysteine. H358 cells were treated for 1 hr with 400 μM H₂O₂ in absence or presence of 20 mM of the ROS-scavenger *N*-acetyl-L-cysteine (NAC). Cell lysates were harvested for western blot analysis and probed with the indicated antibodies.

calcium concentrations after treatment with sorafenib promote ROS production in the mitochondria.²⁶ The importance of ROS for sorafenib induced cell death, was also shown by Coriat *et al.* who found that sorafenib effectiveness in HCC cell lines *in vitro* is mediated by ROS production. Even more interestingly, they showed in sera from HCC patients that in response to sorafenib HCC cancer cells produce massive amounts of ROS, which induce tumor cell death. No or weak ROS production predicts a lack of sorafenib effectiveness.²³ Interestingly, we could link both calcium mobilization and ROS production by sorafenib to a single molecular effector mechanism, namely the activation of AMPK by either CAMKK2 or LKB1. It was recently reported by Fumarola *et al.* that sorafenib promotes an early perturbation of mitochondrial function in breast cancer cells.²⁷ As a response to this stress condition, AMPK was rapidly activated in the cell lines analyzed. Their data also suggest a key role of AMPK-mediated mTORC1 inhibition in the antitumor activity of sorafenib in breast cancer. The modulation of AMPK by sorafenib in combination with everolimus has been reported in osteosarcoma cells, where the activation is mediated by ROS.²⁸ Eum *et al.* described the sustained activation of AMPK by sorafenib in v-Ha-ras-transformed 3T3 cells.²⁹ We show that the AMPK activation is also seen with fluorosorafenib (regorafenib).

Our observation that sorafenib in combination with metformin improves DCR in NSCLC patients was unexpected. However, the number of patients using metformin in our study was small and the observation therefore needs further clinical validation. Although additional mechanisms may be involved, we show that increasing AMPK activity by combining sorafenib with AMPK activators can increase the antiproliferative effects of sorafenib. The antiproliferative effect with the combination of metformin and sorafenib *in vitro* was recently reported for intrahepatic cholangiocarcinoma cells.³⁰ One of the AMPK activators used in our *in vitro* studies was salicylate, the active metabolite of aspirin. The antiproliferative effect that we have seen with the combinations of sorafenib and salicylate is stronger than the effect of the combinations of sorafenib and metformin. A possible explanation for this could be the different mechanisms of AMPK activation by salicylate and metformin. Salicylate binds directly to AMPK α at the same site as the synthetic AMPK activator A-769662 to cause allosteric activation and inhibition of dephosphorylation of the activating phosphorylation site Thr172.¹³ This inhibition of Thr172 dephosphorylation by salicylate will further support sorafenib-mediated AMPK activation. The effects of metformin on AMPK activation are *indirect* and it is therefore unclear if metformin can also inhibit Thr172 dephosphorylation.

The plasma salicylate concentrations in humans treated with oral salsalate³¹ or high-dose aspirin (around 7 g/day)³² are 1-2 mM and accordingly in the concentration range that we used in our *in vitro* experiments with salicylate. The concentrations of metformin used in our *in vitro* and *in vivo*

experiments are equal or lower compared to concentrations used in previous studies^{8,15,19,20,33}, but higher than the recommended therapeutic doses in humans. It is, therefore, difficult to extrapolate our results to the potential effects of metformin in a clinical trial with standard doses metformin. For this reason, the related and more potent-but toxic-drug phenformin is used in some mouse studies.³³ However, it has been reported that AMPK-mediated effects of metformin can be seen *in vivo* with doses comparable to the dose we used *in vivo*.²⁰ Furthermore, our finding that metformin users in a phase II clinical trial with sorafenib have an improved DCR compared to nonmetformin users suggests that the effects can be seen with therapeutic doses of metformin, as was shown before.¹⁸ However, the diabetic patients in our study were using metformin for a long period before administration of sorafenib and the effect can therefore be different for non-diabetic patients starting with metformin in combination with sorafenib.

Our study also highlights the importance of carefully monitoring comedications that are used in clinical trials with anticancer agents, because these medications may influence the response to the drug of interest.

The possible tumor growth acceleration by metformin monotherapy, as observed in our xenograft study, could have clinical implications, as patients with NSCLC treated with metformin for their type II diabetes are at risk for this acceleration. This detrimental effect of metformin on lung cancer has already been described in a large medical report study by Mazzone *et al.*³⁴ They first conclude that the use of metformin is associated with a lower likelihood of developing lung cancer in diabetic patients. However, diabetics who developed lung cancer while receiving metformin were more likely to present with metastatic disease and a shorter survival from the time of diagnosis (Hazard ratio 1.47; 95% CI 1.12–1.92; $p = 0.005$). Acceleration of tumor growth by metformin has also been described for *BRAF*^{V600E}-driven melanomas—but not for *NRAS* mutant melanomas—by Martin *et al.*²⁰ They found that transcriptional upregulation of *VEGFA* via AMPK was responsible for the tumor growth acceleration. We tested in our NSCLC cell lines whether transcriptional *VEGFA* upregulation occurred *in vitro*. We observed a slight (up to twofold) upregulation of *VEGFA* mRNA after 48-hr treatment with 1–3 mM metformin, independent of *LKB1* or *KRAS* mutation status (Supporting Information Figs. 3a–3c). We also analyzed the number of CD31-positive vessels in the A549 xenograft tumor sections from the different treatment groups (Supporting Information Fig. 3d). Tumors treated with metformin monotherapy had a significant increase in the number of vessels compared to vehicle ($p = 0.021$), sorafenib monotherapy ($p < 0.001$) or the combination ($p < 0.001$), indicating that the *VEGFA* upregulation leads to an increase in tumor vessel density *in vivo*.

A study by Shackelford *et al.* demonstrated that phenformin was selectively effective in *LKB1*-deficient NSCLC animal

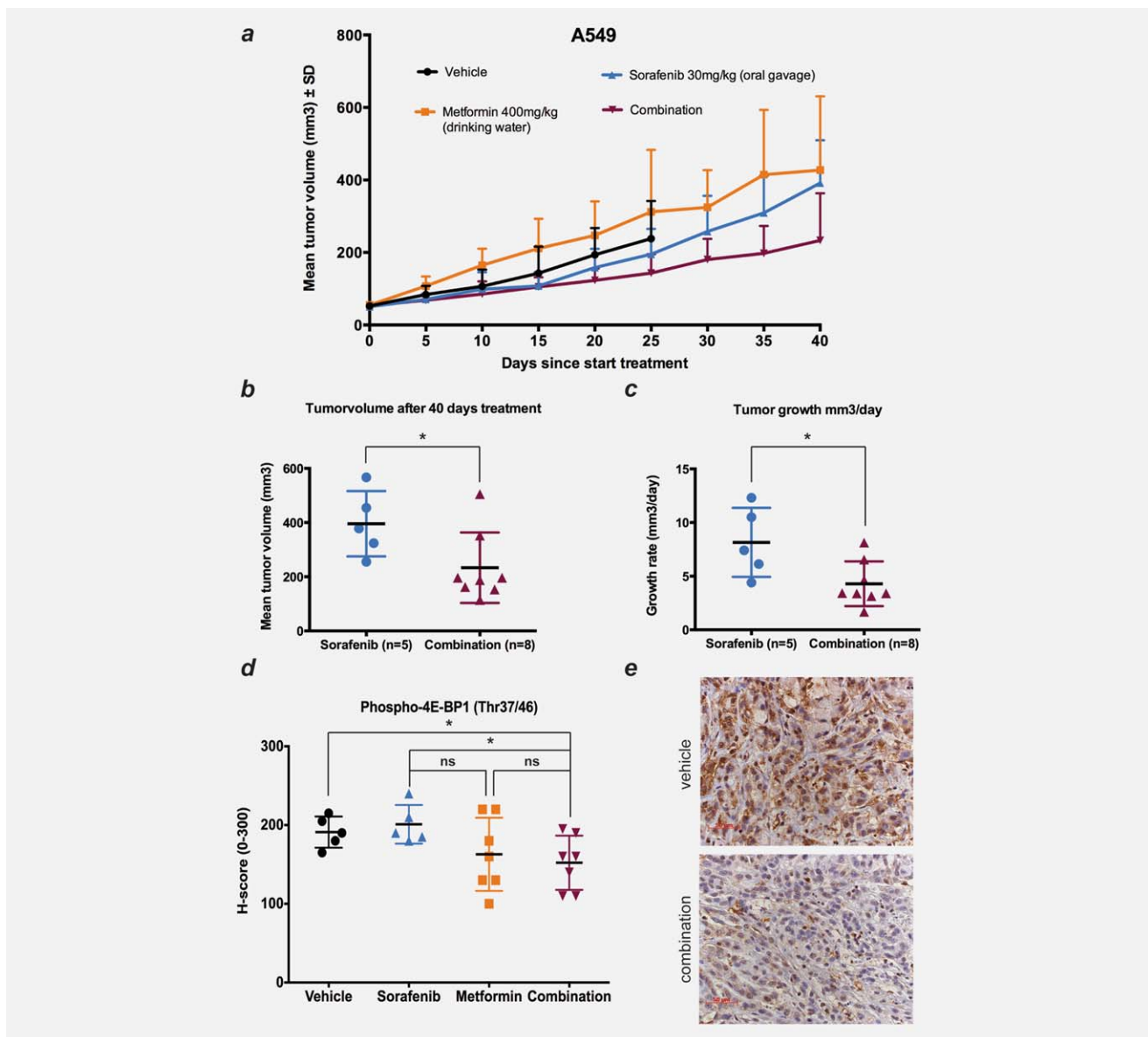


Figure 5. Effect of the combination of sorafenib with metformin in a tumor xenograft model. *a*, The growth curves of A549 cells as tumor xenografts in nude mice treated with vehicle (black curve), sorafenib (blue curve), metformin (yellow curve) or the combination (purple curve). Error bars represent SD; $n = 5-8$. The black curve (vehicle) is shown until 25 days after start treatment, because of a bacterial infection outbreak in this group. *b*, Mean tumor volume (mm³) after 40 days treatment with sorafenib (blue dots; $n = 5$) or the combination of sorafenib with metformin (purple triangles; $n = 8$). A p -value < 0.05 was considered as statistically significant (*). *c*, Mean tumor growth rate (mm³/day), calculated by linear regression modeling of the individual growth curves, for the sorafenib treated tumor xenografts (blue dots; $n = 5$) and the tumor xenografts treated with the combination of sorafenib with metformin (purple triangles; $n = 8$). A p -value < 0.05 was considered as statistically significant (*). *d*, H-score of phospho-4E-BP1 (Thr37/46) immunostaining of A549 xenograft tumor sections of the different treatment groups. p -values < 0.05 were considered as statistically significant (*); ns = nonsignificant. *e*, Images of A549 xenograft tumor sections of vehicle (top) and combination-treated (bottom) tumors immunostained for phospho-4E-BP1 (400 \times magnification).

models.³³ We showed that the synergy between metformin and sorafenib is also seen in *LKB1*-mutant NSCLC cells, which use CAMKK2 to activate AMPK. We did not test the effect of phenformin in combination with sorafenib, but it could well be that the effect in *LKB1*-deficient cells is larger when phenformin is used. The sensitivity of cells to bigua-

nides might also be dependent on the glucose concentration. Birsoy *et al.* showed that cells with defective mitochondrial oxidative phosphorylation, as a result of mitochondrial DNA mutations or impaired glucose utilization, were selectively sensitive to biguanides under metabolic stress conditions.³⁵ For this reason, it is important to state that all the cell lines

used in our experiments were grown in high-glucose medium.

Our data provide a rationale for a clinical trial combining sorafenib and metformin in NSCLC or other cancer types in which sorafenib is either approved (HCC, renal cell carcinoma) or under clinical investigation. The fact that metformin is already used extensively in the clinic with minimal

side effects together with the favorable therapeutic range of metformin makes it relative easy to start these clinical trials.

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References

- Mihaylova MM, Shaw RJ. The AMPK signaling pathway coordinates cell growth, autophagy and metabolism. *Nat Cell Biol* 2011;13:1016–23.
- Hemminki A, Markie D, Tomlinson I, et al. A serine/threonine kinase gene defective in Peutz-Jeghers syndrome. *Nature* 1998;391:184–7.
- Ding L, Getz G, Wheeler DA, et al. Somatic mutations affect key pathways in lung adenocarcinoma. *Nature* 2008;455:1069–75.
- Gill RK, Yang SH, Meerzaman D, et al. Frequent homozygous deletion of the LKB1/STK11 gene in non-small cell lung cancer. *Oncogene* 2011;30:3784–91.
- Evans JM, Donnelly LA, Emslie-Smith AM, et al. Metformin and reduced risk of cancer in diabetic patients. *BMJ* 2005;330:1304–5.
- Dowling RJ, Niraula S, Stambolic V, et al. Metformin in cancer: translational challenges. *J Mol Endocrinol* 2012;48:R31–R43.
- Pierotti MA, Berrino F, Gariboldi M, et al. Targeting metabolism for cancer treatment and prevention: metformin, an old drug with multifaceted effects. *Oncogene* 2013;32:1475–87.
- Ben Sahra I, Le Marchand-Brustel Y, Tanti JF, et al. Metformin in cancer therapy: a new perspective for an old antidiabetic drug? *Mol Cancer Ther* 2010;9:1092–9.
- Huang X, Wullschleger S, Shpiro N, et al. Important role of the LKB1-AMPK pathway in suppressing tumorigenesis in PTEN-deficient mice. *Biochem J* 2008;412:211–21.
- Hardie DG. The LKB1-AMPK pathway—friend or foe in cancer? *Cancer Cell* 2013;23:131–2.
- Fryer LG, Parbu-Patel A, Carling D. The Antidiabetic drugs rosiglitazone and metformin stimulate AMP-activated protein kinase through distinct signaling pathways. *J Biol Chem* 2002;277:25226–32.
- Sun W, Lee TS, Zhu M, et al. Statins activate AMP-activated protein kinase in vitro and in vivo. *Circulation* 2006;114:2655–62.
- Hawley SA, Fullerton MD, Ross FA, et al. The ancient drug salicylate directly activates AMP-activated protein kinase. *Science* 2012;336:918–22.
- Liao X, Lochhead P, Nishihara R, et al. Aspirin use, tumor PIK3CA mutation, and colorectal-cancer survival. *N Engl J Med* 2012;367:1596–606.
- Chen G, Xu S, Renko K, Derwahl M. Metformin inhibits growth of thyroid carcinoma cells, suppresses self-renewal of derived cancer stem cells, and potentiates the effect of chemotherapeutic agents. *J Clin Endocrinol Metab* 2012;97:E510–E20.
- Hirsch HA, Iliopoulos D, Tsiachlis PN, et al. Metformin selectively targets cancer stem cells, and acts together with chemotherapy to block tumor growth and prolong remission. *Cancer Res* 2009;69:7507–11.
- Iliopoulos D, Hirsch HA, Struhl K. Metformin decreases the dose of chemotherapy for prolonging tumor remission in mouse xenografts involving multiple cancer cell types. *Cancer Res* 2011;71:3196–201.
- Jiralerspong S, Palla SL, Giordano SH, et al. Metformin and pathologic complete responses to neoadjuvant chemotherapy in diabetic patients with breast cancer. *J Clin Oncol* 2009;27:3297–302.
- Rocha GZ, Dias MM, Ropelle ER, et al. Metformin amplifies chemotherapy-induced AMPK activation and antitumoral growth. *Clin Cancer Res* 2011;17:3993–4005.
- Martin MJ, Hayward R, Viros A, et al. Metformin accelerates the growth of BRAF V600E-driven melanoma by upregulating VEGF-A. *Cancer Discov* 2012;2:344–55.
- Dingemans AM, Mellema WW, Groen HJ, et al. A Phase II Study of Sorafenib in Patients with Platinum-Pretreated, Advanced (Stage IIIb or IV) Non-Small Cell Lung Cancer with a KRAS Mutation. *Clin Cancer Res* 2013;19:743–51.
- Chiou JF, Tai CJ, Wang YH, et al. Sorafenib induces preferential apoptotic killing of a drug- and radio-resistant Hep G2 cells through a mitochondria-dependent oxidative stress mechanism. *Cancer Biol Ther* 2009;8:1904–13.
- Coriat R, Nicco C, Chereau C, et al. Chausade S, Goldwasser F, Batteux F. Sorafenib-induced hepatocellular carcinoma cell death depends on reactive oxygen species production in vitro and in vivo. *Mol Cancer Ther* 2012;11:2284–93.
- Mungai PT, Waypa GB, Jairaman A, et al. Hypoxia triggers AMPK activation through reactive oxygen species-mediated activation of calcium release-activated calcium channels. *Mol Cell Biol* 2011;31:3531–45.
- Alexander A, Cai SL, Kim J, et al. ATM signals to TSC2 in the cytoplasm to regulate mTORC1 in response to ROS. *Proc Natl Acad Sci USA* 2010;107:4153–8.
- Rahmani M, Davis EM, Crabtree TR, et al. The kinase inhibitor sorafenib induces cell death through a process involving induction of endoplasmic reticulum stress. *Mol Cell Biol* 2007;27:5499–513.
- Fumarola C, Caffarra C, La Monica S, et al. Effects of sorafenib on energy metabolism in breast cancer cells: role of AMPK-mTORC1 signaling. *Breast Cancer Res Treat* 2013;141:67–78.
- Pignochino Y, Dell'Aglio C, Basirico M, et al. The Combination of Sorafenib and Everolimus Abrogates mTORC1 and mTORC2 upregulation in osteosarcoma preclinical models. *Clin Cancer Res* 2013;19:2117–31.
- Eum KH, Ahn SK, Kang H, et al. Differential inhibitory effects of two Raf-targeting drugs, sorafenib and PLX4720, on the growth of multidrug-resistant cells. *Mol Cell Biochem* 2013;372:65–74.
- Ling S, Feng T, Ke Q, et al. Wang L. Metformin inhibits proliferation and enhances chemosensitivity of intrahepatic cholangiocarcinoma cell lines. *Oncol Rep* 2014;31:2611–8.
- Fleischman A, Shoelson SE, Bernier R, et al. Salicylate improves glycemia and inflammatory parameters in obese young adults. *Diabetes Care* 2008;31:289–94.
- Hundal RS, Petersen KF, Mayerson AB, et al. Mechanism by which high-dose aspirin improves glucose metabolism in type 2 diabetes. *J Clin Invest* 2002;109:1321–6.
- Shackelford DB, Abt E, Gerken L, et al. LKB1 Inactivation dictates therapeutic response of non-small cell lung cancer to the metabolism drug phenformin. *Cancer Cell* 2013;23:143–58.
- Mazzone PJ, Rai H, Beukemann M, et al. The effect of metformin and thiazolidinedione use on lung cancer in diabetics. *BMC Cancer* 2012;12:410.
- Birsoy K, Possemato R, Lorbeer FK, et al. Metabolic determinants of cancer cell sensitivity to glucose limitation and biguanides. *Nature* 2014;508:108–12.