Published in final edited form as:

ChemMedChem. 2015 November ; 10(11): 1915–1923. doi:10.1002/cmdc.201500371.

Development of Potent Adenosine Monophosphate-Activated Protein Kinase Activators

Eman M. E. Dokla^{a,b}, Chun-Sheng Fang^a, Po-Ting Lai^a, Dr. Samuel K. Kulp^a, Dr. Rabah A.T. Serya^b, Dr. Nasser S. M. Ismail^b, Prof. Khaled A. M. Abouzid^{b,*}, and Prof. Ching-Shih Chen^{a,c,*}

^aRm 336, Parks Hall, Division of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, The Ohio State University, 500 West 12th Avenue, Columbus, OH 43210, USA

^bPharmaceutical Chemistry Department, Faculty of Pharmacy, Ain Shams University, Abbassia, Cairo, Egypt

^cInstitute of Biological Chemistry, Academia Sinica, Taipei, Taiwan

Abstract

Previously, we reported the identification of a thiazolidinedione-based adenosine monophosphate-activated protein kinase (AMPK) activator, **1** (N-[4-({3-[(1-methylcyclohexyl)methyl]-2,4-dioxothiazolidin-5-ylidene)methyl}phenyl)-4-nitro-3-(trifluoromethyl)benzenesulfonamide), which provided a proof-of-concept to delineate the intricate role of AMPK in regulating oncogenic signaling pathways associated with cell proliferation and epithelial-mesenchymal transition (EMT) in cancer cells. In this study, we used **1** as a scaffold to conduct lead optimization, which generated a series of derivatives. Analysis of the anti-proliferative and AMPK-activating activities of individual derivatives revealed a distinct structure-activity relationship and identified **59** (N-(3-Nitrophenyl)-N'-{4-[(3-{[3,5-bis(trifluoromethyl)phenyl]methyl}-2,4-dioxo-5-thiazolidinylidene)methyl]phenyl}-urea) as the optimal agent. Relative to **1**, **59** exhibited multifold higher potency in upregulating AMPK phosphorylation in multiple cell lines irrespective of their liver kinase B1 (LKB1) functional status, accompanied by parallel changes in the phosphorylation/expression levels of p70S6K, Akt, Foxo3a, and EMT-associated markers. Consistent with its predicted activity against tumors with activated Akt status, oral **59** was efficacious in suppressing the growth of phosphatase and tensin homolog (PTEN)-null PC-3 xenograft tumors in nude mice. Together, these findings suggest that **59** has clinical value in therapeutic strategies for PTEN-negative cancer and warrants continued investigation in this regard.

Graphical Abstract

Keywords

Antitumor agents; AMPK activator; Drug Discovery; Lead optimization; Medicinal Chemistry

Introduction

Adenosine monophosphate-activated protein kinase (AMPK) is a key player in maintaining energy homeostasis in response to metabolic stress.^[1–4] Beyond diabetes and metabolic syndrome,^[5] there is a growing interest in the therapeutic exploitation of the AMPK pathway in cancer treatment in light of its unique ability to regulate cancer cell proliferation through the reprogramming of cell metabolism.^[6–8] This premise was corroborated by the ability of the antidiabetic drug metformin, which acts by disrupting mitochondrial respiration to activate AMPK, to lower cancer risk and to improve outcomes of many types of cancer.^[9, 10] In addition to cell metabolism, recent evidence suggests that AMPK plays an intricate role in regulating cancer cell proliferation, survival, and invasive phenotype by targeting multiple oncogenic signaling pathways, including those mediated by Akt, mTOR, and MDM2.^[11–13] Consequently, the past few years have witnessed the development of a number of novel AMPK activators as discussed in several reviews^[14–16], including A-769662,^[17] PT-1,^[18] OSU-53 (**1**; Figure 1),^[19] (*E*)-3-((3-[(4-chlorophenyl)(phenyl)methylene]-2-oxoindolin-1-yl)methyl)benzoic acid,^[20] and 991.^[14, 21] Mechanistically, these direct activators circumvent the dependence on liver kinase B1 (LKB1), an upstream kinase of AMPK under conditions of energy stress, which is often inactivated in cancer cells through mutations in the course of tumorigenesis.^[13]

Previously, based on the screening of a thiazolidinedione-based focused compound library, we identified **1** as a direct AMPK activator in LKB1-deficient MDA-MB-231 cells.^[19] **1** served as a proof-of-concept compound to delineate the intricate mechanisms by which AMPK regulates cell proliferation and epithelial-mesenchymal transition (EMT) in cancer cells,^[11, 12] among which the ability of **1** to target the Akt-MDM2-Foxo3a signaling pathway is especially noteworthy. Specifically, pharmacological activation of AMPK leads

to protein phosphatase 2A-facilitated Akt dephosphorylation. This Akt inactivation results in reduced phosphorylation of the E3 ligase murine double minute 2 (MDM2) and its substrate Foxo3a, which facilitates the cytoplasmic sequestration and nuclear translocation of MDM2 and Foxo3a, respectively. The physical separation of Foxo3a from MDM2 increases Foxo3a protein stability and accumulation.

These findings underscore the translational potential of targeting AMPK activation as a therapeutic strategy for metastatic cancer treatment, thereby providing a rationale to use **1** as a scaffold to conduct lead optimization. This effort has netted a series of potent AMPK activators, among which **59** represented the optimal agent. Relative to **1**, **59** exhibited multifold higher potency in facilitating AMPK activation, accompanied by parallel changes in Akt phosphorylation and Foxo3a induction, in MDA-MB-231 and other cancer cell lines. Equally important, **59** was orally active in suppressing xenograft tumor growth in nude mice without incurring acute toxicity.

Results

Structural optimization of **1**

We carried out modeling analysis by docking **1** into the recently released x-ray crystal structure of human AMPK (PDB ID, 4CFE).^[22] This analysis revealed that **1** bound to key amino acids in the allosteric site formed at the interface between the kinase domain and carbohydrate-binding module (CBM) through the following forces, as depicted in Figure 1A.

- a. Hydrophobic interactions between the cyclohexane/benzylidene moieties and the large hydrophobic pocket formed by residues Val11, Leu18, Ile46, and Leu47 (kinase domain) and Val81, Val 113, and Val118 (CBM domain);
- b. Cation- π interactions between the thiazolidinedione ring and Lys31 (kinase domain), as well as between the benzylidene ring and Arg83 (CBM);
- c. Hydrogen bonding between the -SO₂NH- moiety and Lys29 and Asp88, and between the -NO₂ substituent and Lys29. Based on this docking analysis, we employed the following strategies to conduct the structural modification of **1** (Figure 1B). General procedures for the synthesis of these compounds are depicted in Figure 1C. The initial screening was based on the activities of individual compounds, each at 5 μ M, in suppressing the viability of LKB1-deficient MDA-MB-231 cells by using MTT assays.

Step 1 (Series A): the methylcyclohexyl group of **1** was replaced with various substituted phenyl rings to exploit the hydrophobic pocket. Among thirty-five derivatives (**2** – **36**) examined (Figure 2), **16** (3,5-di-CF₃-phenyl) and **26** [4-(methylthio)phenyl] exhibited moderately higher antiproliferative potency as compared to **1** (Figure 3A).

Step 2 (Series B): the 3-CF₃-4-NO₂-phenyl ring of **16** and **26** were replaced with a 4-Cl-3-NO₂- or 4-Cl-2-NO₂-phenyl moiety to generate **37** – **39** (Figure 2). As shown, substitution of the 3-CF₃-4-NO₂-phenyl moiety of **16** and **26** with 4-Cl-3-NO₂-

substantially increased the antiproliferative activities of the resulting compounds (**37** and **39**) (Figure 3A). Relative to **37**, the antiproliferative potency of **38** was attenuated as a result of the different configuration of the $-\text{NO}_2$ function on the phenyl ring, indicating its distinct role in interacting with the binding motif. Moreover, **40**, a regioisomer of **16**, was synthesized and assessed for its *in vitro* antiproliferative activity, which, however, was abolished by the change in the regiochemical configuration of the terminal phenyl-sulfonamide substructure.

Step 3 (Series C - E): as bioisosteric replacement represents a common strategy for lead optimization,^[23, 24] we tested the effects of replacing the sulfonamide linker with amide (Series C, **41** and **42**; Series D; **43** – **50**), urea (Series E; **51** – **67**, **73** – **75**, and **78**), and thiourea (Series E; **68** – **72**, **76**, and **77**) in conjunction with varying the terminal substituted phenyl moiety on antiproliferative efficacy (Figure 2). This strategy generated an array of derivatives with the ability to suppress cell viability by 75% at 5 μM , which included **43**, **46** – **48**, **50** – **54**, **59**, **62**, **68**, **69**, **71**, **74**, and **75** (Figure 3A). A unique feature common to many of these compounds is the 3- NO_2 -phenyl moiety, with the exception of **71**, in which the 3-nitro-substituent was replaced by a sulfonamide function.

Step 4 (Series F and G): continued structural modifications of the two optimal derivatives **51** and **59** generated Series F (**79** – **84**) and G (**85**, **86**), respectively (Figure 2). Substitution on the benzylidene moiety of **51** with different functionalities, including $-\text{Cl}$, $-\text{CF}_3$, and $-\text{OCH}_3$, at the C-2' and, to a great extent, C-3' positions diminished the antiproliferative efficacy relative to the parent compound (Figure 3A), indicating the distinct stereoelectronic effect of these substituents. In particular, addition of a chlorine atom to the C-2' position completely abolished the activity. Replacement of the phenyl ring of the benzylidene moiety of **59** with a pyridine and/or replacing the terminal 3,5-di- CF_3 -phenyl with 4-trifluoromethyl-phenyl (**85** and **86**, respectively) did not show improved potency relative to **59**.

Identification of optimal AMPK activators via cell-based assays

Of the eighty-five derivatives examined, twenty-one compounds were effective in suppressing the viability of MDA-MB-231 cells by 75% at 5 μM after 72 h of exposure, including **37**, **39**, **43**, **46** – **48**, **50** – **54**, **59**, **62**, **68**, **69**, **71**, **74**, **75**, **80**, **85**, and **86** (Figure 3A, indicated by *). We further assessed the effects of these compounds vis-à-vis **1** on the phosphorylation status of AMPK in MDA-MB-231 cells by Western blot analysis. Due to lack of LKB1, MDA-MB-231 cells were resistant to the effect of metformin-induced metabolic stress on AMPK activation (Figure 3B, left), reminiscent to that previously reported.^[25] In addition, these cells were relatively insensitive to 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR)-facilitated AMPK phosphorylation (right). In contrast, these derivatives exhibited differential effects on AMPK phosphorylation, with **59** showing the highest potency, among those with high activity, including **51**, **53**, **69**, **71**, **74**, and **86** (Figure 3C). Based on these data, **59**, its close derivative **51**, and **71** were selected for further evaluations vis-à-vis **1**.

MTT assays revealed a positive correlation between the antiproliferative efficacies of these compounds (IC₅₀ values at 72 h: **59**, 1.8 μ M; **51**, 3.5 μ M; **71**, 4 μ M; **1**, 6 μ M) (Figure 4A) and their aforementioned abilities to upregulate AMPK phosphorylation (Figure 3C).

This observation was confirmed by the differences in their dose-dependent effects on AMPK phosphorylation, which was accompanied by parallel increases in the phosphorylation of its downstream substrate acetyl-CoA carboxylase (ACC) (Figure 4B). While **59** was effective in inducing AMPK phosphorylation at 1 μ M, other compounds examined would require substantially higher concentrations (**1**, 7.5 μ M; **51**, 2.5 μ M; **71**, 2.5 – 5 μ M) to achieve the same level of increase in AMPK phosphorylation.

Mechanistic interrogation of the effects of **51**, **59**, and **71** on AMPK signaling

It is well recognized that AMPK acts as an energy sensor by mediating the phosphorylating inactivation of ACC and other key lipogenic enzymes in response to cellular AMP or ADP elevation.^[1] Equally important, we previously used **1** as a proof-of-concept compound to demonstrate the suppressive effect of AMPK activation on multiple oncogenic signaling pathways, including those mediated by Akt and mTOR/p70S6K.^[11, 12] Consequently, as part of the mechanistic interrogation of **59**, **51**, and **71**, we compared their effects versus those of **1** on these aforementioned signaling markers. As shown, **59** (0.3 – 2.5 μ M) and, to a slightly lesser extent, **51** (0.5 – 5 μ M) were effective in increasing the phosphorylation of AMPK and its target ACC, accompanied by parallel decreases in the phosphorylation of p70S6K, a marker of mTOR activity, and Akt in a manner reminiscent of that of **1** (2.5 – 10 μ M) in MDA-MB-231 cells (Figure 4B). In contrast, **71**, unlike **59** and **51**, was ineffective in downregulating the phosphorylation of p70S6K and Akt, suggesting a clear structure-activity relationship (SAR) among these three AMPK-activating agents in modulating AMPK downstream effectors (See the following section for further discussion). This distinct SAR was further manifested by **60** that differs from **59** in the substitution position of the –NO₂ function (C-4 versus C-3). Despite largely shared structural motifs, **60** exhibited substantially lower efficacy, relative to **59**, in suppressing the viability (IC₅₀, 6 μ M versus 1.8 μ M for **59**, data not shown) and eliciting AMPK activation (Figure 4C) in MDA-MB-231 cells.

To refute the possibility that the ability of **59** to activate AMPK signaling was unique to MDA-MB-231 cells, we expanded our investigation to two additional cell lines, MDA-MB-468 breast cancer cells and phosphatase and tensin homologue (PTEN)-null PC-3 prostate cancer cells. MTT assays indicate that the antiproliferative efficacies of **1** and **59** in MDA-MB-468 and PC-3 cells were similar to those in MDA-MB-231 cells with the respective IC₅₀ values as follows: **1**, 5.8 and 6.2 μ M; **59**, 1.5 and 1.6 μ M (data not shown). More important, **1** and **59** affected AMPK phosphorylation and various AMPK downstream markers in these two cell lines in a manner similar to that in MDA-MB-231 cells (Figure 5A & B), indicating these effects were not a cell line-specific phenomenon. Moreover, consistent with our previous results demonstrating the role of AMPK in suppressing EMT via Foxo3a upregulation,^[12] exposure of MDA-MB-231 and PC-3 cells to **59** dose-dependently increased Foxo3a expression, leading to the reversal of their mesenchymal phenotype, as manifested by increases in the epithelial markers claudin-1 in MDA-MB-231

cells, which are deficient in E-cadherin, and E-cadherin in PC-3 cells, in conjunction with parallel decreases in the mesenchymal markers vimentin and Snail in both cell lines (Figure 5C).

59 is not a PAINS (Pan Assay Interference compounds) compound

It has recently been reported that compounds with certain classes of chemically reactive substructures could give rise to false-positive results by interfering with assays through covalent modifications, metal chelation, photometric interference, redox activity, and/or aggregate formation.^[26–28] These interferences might lead to false leads that could not be optimized. Families of these compounds, classified as PAINS, include alkylidene-containing heterocycles, hydroquinones, catechols, hydroxyphenylhydrozones, Mannich bases of phenols, and 2-amino-3-carbonyl thiophenes. Although **59** was not recognized by the PAINS filters (<http://cbligand.org/PAINS/login.php>),^[27] it contains an alkylidene thiazolidinedione substructure, which could potentially serve as a Michael acceptor to covalently modify the target protein AMPK upon binding. This possibility was refuted by the distinct SAR displayed by various close derivatives of **59** in activating AMPK, as demonstrated in Figure 3 & 4. Moreover, we obtained two additional lines of evidence to support that **59** is not a PAINS compound. First, compound **87**, a saturated counterpart of compound **59**, retained the ability to suppress cell viability (IC₅₀, 9 μ M, data not shown) and to activate AMPK, though with lower potency, in MDA-MB-231 cells (Figure 6A).

The lower efficacy of **87** in eliciting AMPK activation might arise from loss of the rigid conformation associated with the removal of the double bond, resulting in weaker interactions with the binding motif. Second, we conducted a washout experiment, in which **59** was removed from the culture medium after 12 h of drug exposure. In principle, if the mode of AMPK activation by **59** was mediated through the formation of covalent adducts, the course of enzyme activation would not be adversely affected by the drug removal. However, this washout experiment indicated that AMPK phosphorylation in drug-treated MDA-MB-231 cells returned to the basal level following the removal of **59** from the medium (Figure 6B). This rapid restoration of AMPK phosphorylation suggests a reversible nature of this ligand-protein interaction.

***In Vivo* Efficacy of 59 versus 1 in Suppressing PC-3 Xenograft Tumor Growth**

Based on the above findings, we selected **59** as the lead AMPK activator vis-à-vis **1** for the evaluation of *in vivo* efficacy. In light of the ability of **59** to target the AMPK-Akt signaling axis, we examined **59** versus **1** as a single agent against the growth of subcutaneous xenograft tumors established from PTEN-null PC-3 cells in athymic nude mice (n = 7 for each group). As shown, daily administration of **59** at 50 mg/kg via oral gavage significantly suppressed PC-3 xenograft tumor growth after 28 days of treatment (65% inhibition, $P = 0.0003$ relative to vehicle-treated control) (Figure 7A, left).

In contrast, **1** gave rise to modest, but statistically not significant inhibition (36% inhibition; $P = 0.069$). Equally important, **59** was well tolerated as no significant loss of body weight was noted (Figure 7A, right). Moreover, Western blot analysis of tumor lysates showed that this tumor-suppressive activity correlated with the ability of **59** to activate AMPK signaling,

as manifested by parallel changes in the phosphorylation levels of AMPK, ACC, and Akt (Figure 7B). As compared to **1**, **59** elicited a greater extent of AMPK activation in tumors.

Discussion

Using **1** as a proof-of-concept, we have previously demonstrated the relevance of AMPK activation as a therapeutic strategy to suppress tumorigenesis and invasive phenotype by targeting multiple oncogenic pathways.^[11, 12] However, **1** exhibited modest potency in facilitating AMPK activation, which underlies the impetus of lead optimization of **1** to develop more potent AMPK activators. Based on modeling analysis, we modified different substructures of compound **1** in a stepwise manner, as outlined in Figure 1B, to generate a total of eighty-five derivatives, among which **59** represented the lead agent. Analysis of the anti-proliferative and/or AMPK-activating activities of individual derivatives revealed a distinct SAR. Among various functionalities that were modified, the linker between the two aromatic systems and the NO₂ substituent on the phenyl ring had the most profound effects on the aforementioned activities. Among the twenty-one derivatives selected for Western blot analysis (Figure 3C), compounds with a urea linker (**51–54**, **59**, **62**, **68**, **69**, **71**, **74**, **80**, **85**, **86**), in general, showed superior potency in facilitating AMPK phosphorylation as compared to those containing sulfonamide (**37**, **39**) or amide (**43**, **46–48**, **50**) (Figure 3C). Moreover, shifting the orientation of the NO₂ function of **59** (C-3 → C-4) substantially attenuated the antiproliferative and AMPK-activating activities of the resulting compound **60**. This role of the –NO₂ substituent in mediating AMPK activation was also manifested by the differential activity between **51** and **59**. Relative to **59**, **51** showed lower activities in anti-proliferation and AMPK activation, which might be attributable to the stereochemical effect of the additional chlorine atom on protein binding. Together, these data suggest intricate interplay between the –NO₂ substituent on the terminal phenyl ring and neighboring functionalities in interacting with the binding pocket of AMPK enzyme. Compound **59** exhibited multifold higher potency than **1** in upregulating the phosphorylation of AMPK and its downstream target ACC in multiple cell lines irrespective of their LKB1 functional status. Equally important, this AMPK activation was accompanied by parallel changes in the phosphorylation/expression levels of p70S6K, Akt, Foxo3a, and various EMT-associated markers, confirming its ability to target the AMPK-Akt-Foxo3a signaling axis.

Our previous study has demonstrated the *in vivo* efficacy of **1** in suppressing PTEN-functional MDA-MB-231 xenograft tumor growth.^[11] In light of its predicted activity against tumors with activated Akt status, we examined the tumor-suppressive activity of **59** versus **1** (50 mg/kg daily via oral gavage) in PTEN-null PC-3 tumor-bearing athymic nude mice. Consistent with the relative *in vitro* anti-proliferative potency, **59** showed higher *in vivo* efficacy than **1** in inhibiting PC-3 xenograft tumor growth, which correlated with their respective activities in facilitating AMPK activation in tumors. Together, these findings suggest that **59** has clinical value in therapeutic strategies for PTEN-negative cancer and warrants continued investigation in this regard.

PAINS have become a major issue in drug discovery because they give rise to false-positive results in enzyme-based assays due to their confounding properties, which would lead to problematically flat SAR if the chemically reactive substructure remains unchanged.^[26–28]

In our study, there existed a clear SAR in the course of optimization, and it is noteworthy that disruption of the conjugated system by removing the double bond did not abrogate the ability of the resulting compound, i.e., **87**, to activate AMPK, though with lower potency. In addition, the washout experiment indicates the ligand-protein binding was reversible, which in conjunction with the improved *in vitro* and *in vivo* antitumor efficacy of **59** relative to **1** by targeting the AMPK-Akt signaling axis refutes the possibility that **59** behaved like a PAINS compound.

Conclusion

In light of the demonstrated ability of **1** to target the Akt-Foxo3a signaling pathway via AMPK activation, this study described the lead optimization of **1** to develop a series of potent AMPK activators. This effort netted the lead compound **59** that displayed high *in vitro* and *in vivo* efficacy in activating AMPK and suppressing PTEN-negative tumor growth, which might have translational potential in fostering novel therapeutic strategies for cancer therapy.

Experimental Section

Chemistry

Detailed information on the syntheses of **2** to **87** is described in the **Supporting Information**. Compound **1** was synthesized as previously reported (compound **53** therein).^[19] All commercially available reagents were used without further purification unless otherwise stated. Routine ¹H and ¹³C nuclear magnetic resonance spectra were recorded on the Bruker AV300 or Bruker Ascend 400. Samples were dissolved in deuterated chloroform (CDCl₃) or dimethyl sulfoxide (DMSO-d₆) and tetramethylsilane (TMS) was used as a reference. Electrospray ionization mass spectrometry analyses were performed with a Micromass Q-ToF II high resolution electrospray mass spectrometer in The Ohio State University Campus Chemical Instrument Center. All compounds for bioassay were identified with ¹H NMR, ¹³C NMR and HRMS, and in purity higher than 95%. The purity of all compounds was confirmed by a Hitachi Elite LaChrom HPLC system (comprised of a Versa Grad Prep 36 pump, an L-2400 UV detector, an L-2200 auto sampler and a 150 × 4.6 mm Agilent ZORBAX Eclipse XDB-C18 5μ column; detection, 360 nm). A linear solvent gradient with a mobile phase of 40% water in acetonitrile to 100% acetonitrile in 30 min was used.

Docking analysis

Docking was performed using Discovery Studio CDocker protocol (version 2.5, Accelrys, Inc., San Diego, CA). The X-ray crystal structure of human AMPK enzyme was obtained from PDB server (PDB ID: 4CFE). The protein structure was prepared according to the standard protein preparation procedure integrated in Accelry's Discovery Studio 2.5, which involved adding hydrogen atoms, completing the missing loops and assigning force field parameters. CHARMM force field was used for simulation studies and then the protein structure was minimized using 1000 iterations of steepest descent minimization algorithm. The ligands were drawn using the sketching tools of Accelry's Discovery Studio and they

were prepared for docking by adding hydrogen atoms and partial charges using the Momany-Rone method. For docking, the CDocker protocol was used. The binding site was defined by the residues within 10 Å distance from the co-crystallized ligand. The default values of CDocker were used. Ten different docking solutions were generated and visually inspected for selection of the best binding mode. The docking mode was represented using Chimera software version 1.8.1.^[29]

Cell lines, culture, reagents and antibodies

The MDA-MB-231 and MDA-MB-468 breast cancer and PC-3 prostate cancer cell lines were purchased from American Type Culture Collection (Manassas, VA). Media used for the maintenance of these cells were as follows: MDA-MB-231 and MDA-MB-468, DMEM; PC-3, RPMI 1640, all of which were supplemented with 10% fetal bovine serum. Cells were incubated at 37°C in a humidified incubator containing 5% CO₂. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide] was obtained from TCI America (Portland, OR), and the ECL Western Blotting System from GE Healthcare Life Sciences (Pittsburgh, PA). Antibodies specific for the following protein targets were used: p-Thr172-AMPK, AMPK, p-Thr389-p70S6K, p70S6K, Ser473-Akt, Akt, Foxo3a, claudin-1, vimentin, snail, and β-actin (Cell Signaling Technology, Inc., Beverly, MA); E-cadherin, BD Biosciences (San Diego, CA). Alexa Fluor 555- and 488-conjugated goat anti-rabbit and anti-mouse IgG were purchased from Invitrogen (Carlsbad, CA), and anti-mouse and anti-rabbit secondary antibodies was obtained from Jackson ImmunoResearch Laboratories (West Grove, PA).

Cell viability assay

Cell viability was assessed by using the MTT assay in six replicates. Cells were seeded and incubated in 96-well plates in the respective medium with 10% FBS for 24 h, and then exposed to test agents at the indicated concentrations dissolved in DMSO in 5% FBS-supplemented medium. After 72 h of treatment, the medium was removed and replaced by 200 µL of 0.5 mg/mL MTT in 10% FBS-containing medium, and cells were incubated at 37°C for 2 h. Supernatants were removed, and the MTT dye was solubilized in 120 µL/well of DMSO. Absorbance at 570 nm was determined on a plate reader. Cell viabilities are expressed as percentages of that in the corresponding vehicle-treated control group.

Cell lysis and immunoblotting

Cells were exposed to the test agents in 10-cm dishes and then collected by scraping. The cell pellets were washed once with PBS, and then lysed in SDS lysis buffer containing 50 mM Tris-HCl (pH 8), 10 mM EDTA, 1% SDS, and a commercial protease inhibitor cocktail (2 mM AEBSF, 1 mM EDTA, 130 µM bestatin, 14 µM E-64, 1 µM leupeptin, 0.3 µM aprotinin). After centrifugation of lysates for 20 min at 14,000 × g, and the supernatants were collected. One µL of each supernatant was used for determination of protein concentration using a colorimetric bicinchoninic acid assay (Pierce, Rockford, IL), and to the remaining sample was added an equal volume of 2× SDS-polyacrylamide gel electrophoresis sample loading buffer (62.5 mM Tris-HCl, pH 6.8, 4% SDS, 5% β-mercaptoethanol, 20% glycerol, 0.1% bromophenol blue), followed by incubation in boiling water for 5 min. Equivalent amounts of proteins were resolved in SDS-polyacrylamide gels, and then transferred to nitrocellulose

membranes using a semidry transfer cell. The transblotted membrane was washed twice with Tris-buffered saline containing 0.1% Tween 20 (TBST). After blocking with TBST containing 5% nonfat milk for 40 min, the membrane was incubated with the appropriate primary antibody (1:1000) in TBST-1% nonfat milk at 4°C overnight. The membrane was then washed three times with TBST for a total of 15 min, followed by incubation with goat anti-rabbit or anti-mouse IgG-horseradish peroxidase conjugates (1:2000) for 1 h at room temperature and four washes with TBST for a total of 1 h. The immunoblots were visualized by enhanced chemiluminescence.

Testing of *in vivo* efficacy of 1 and 59 in PC-3 tumor-bearing nude mice

Athymic nude mice (Hsd:Athymic Nude-*Foxn1*^{nu}; 5–6 weeks of age; Harlan, Indianapolis, IN) were group-housed under conditions of constant photoperiod (12 hours light: 12 hours dark) with *ad libitum* access to sterilized food and water. All experimental procedures using mice were done in accordance with protocols approved by the Institutional Animal Care and Use Committee of The Ohio State University (US Office of Laboratory Animal Welfare, Assurance #A3261-01). Ectopic tumors were established in athymic nude mice by subcutaneous injection of PC-3 cells (1×10^6 cells/mouse). The establishment and growth of tumors were monitored by direct measurement with calipers. Mice with established tumors (mean starting tumor volume, approximately 50 mm³) were randomized to three treatment groups (n = 7). Mice were treated once daily for 28 days by oral gavage with (a) **1** at 50 mg/kg body weight, (b) **59** at 50 mg/kg body weight, or (c) vehicle (10% DMSO/0.5% methylcellulose/0.1% Tween 80 [v/v] in sterile water). Tumor burdens were determined weekly using calipers (tumor volume, width² × length × 0.52).

Statistical analysis

In vitro experiments were performed using 3 to 6 replicates in each group. All *in vitro* experiments were carried out at least two times on different occasions. Differences between group means of tumor burden were analyzed for statistical significance using two-sided Student *t* tests. Differences were considered significant at $P < 0.05$.

Acknowledgments

This work was supported by NIH grant R01CA112250 (C.S.C.) and a joint supervision fellowship from the Egyptian government (E.M.E.D.).

Abbreviations Used

ACC	acetyl-CoA carboxylase
AICAR	5-aminoimidazole-4-carboxamide ribonucleotide
AMPK	adenosine monophosphate-activated protein kinase
CBM	carbohydrate-binding module
EMT	epithelial-mesenchymal transition
LKB1	liver kinase B1

MDM2	murine double minute 2
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide
PTEN	phosphatase and tensin homologue

References

1. Kahn BB, Alquier T, Carling D, Hardie DG. Cell Metab. 2005; 1:15–25. [PubMed: 16054041]
2. Long YC, Zierath JR. J Clin Invest. 2006; 116:1776–1783. [PubMed: 16823475]
3. Lim CT, Kola B, Korbonits M. J Mol Endocrinol. 2010; 44:87–97. [PubMed: 19625456]
4. Hardie DG. Genes Dev. 2011; 25:1895–1908. [PubMed: 21937710]
5. Zhang BB, Zhou G, Li C. Cell Metab. 2009; 9:407–416. [PubMed: 19416711]
6. Hardie DG. Annu Rev Pharmacol Toxicol. 2007; 47:185–210. [PubMed: 16879084]
7. Hardie DG. Cold Spring Harb Symp Quant Biol. 2011; 76:155–164. [PubMed: 22071265]
8. Chuang, HC.; Chou, CC.; Kulp, SK.; Chen, CS. Curr Pharm Des. Vol. 20. Netherlands: 2014. p. 2607-2618.
9. Leone A, Di Gennaro E, Bruzzese F, Avallone A, Budillon A. Cancer Treat Res. 2014; 159:355–376. [PubMed: 24114491]
10. Wheaton WW, Weinberg SE, Hamanaka RB, Soberanes S, Sullivan LB, Anso E, Glasauer A, Dufour E, Mutlu GM, Budigner GS, Chandel NS. Elife. 2014; 3:e02242. [PubMed: 24843020]
11. Lee KH, Hsu EC, Guh JH, Yang HC, Wang D, Kulp SK, Shapiro CL, Chen CS. J Biol Chem. 2011; 286:39247–39258. [PubMed: 21917926]
12. Chou CC, Lee KH, Lai IL, Wang D, Mo X, Kulp SK, Shapiro CL, Chen CS. Cancer Res. 2014; 74:4783–4795. [PubMed: 24994714]
13. Shackelford, DB.; Shaw, RJ. Nat Rev Cancer. Vol. 9. England: 2009. p. 563-575.
14. Giordanetto F, Karis D. Expert Opin Ther Pat. 2012; 22:1467–1477. [PubMed: 23136886]
15. Wang, Z.; Huo, J.; Sun, L.; Wang, Y.; Jin, H.; Yu, H.; Zhang, L.; Zhou, L. Curr Comput Aided Drug Des. Vol. 7. United Arab Emirates; 2011. p. 214-227.
16. Rana S, Blowers EC, Natarajan A. J Med Chem. 2015; 58:2–29. [PubMed: 25122135]
17. Cool, B.; Zinker, B.; Chiou, W.; Kifle, L.; Cao, N.; Perham, M.; Dickinson, R.; Adler, A.; Gagne, G.; Iyengar, R.; Zhao, G.; Marsh, K.; Kym, P.; Jung, P.; Camp, HS.; Frevert, E. Cell Metab. Vol. 3. United States; 2006. p. 403-416.
18. Pang, T.; Zhang, ZS.; Gu, M.; Qiu, BY.; Yu, LF.; Cao, PR.; Shao, W.; Su, MB.; Li, JY.; Nan, FJ.; Li, J. J Biol Chem. Vol. 283. United States: 2008. p. 16051-16060.
19. Guh JH, Chang WL, Yang J, Lee SL, Wei S, Wang D, Kulp SK, Chen CS. J Med Chem. 2010; 53:2552–2561. [PubMed: 20170185]
20. Yu LF, Li YY, Su MB, Zhang M, Zhang W, Zhang LN, Pang T, Zhang RT, Liu B, Li JY, Li J, Nan FJ. ACS Med Chem Lett. 2013; 4:475–480. [PubMed: 24900695]
21. Bookser, BC.; Dang, Q.; Gibson, TS.; Jiang, H.; Chung, DM.; Bao, J.; Jiang, J.; Kassick, A.; Kekec, A.; Lan, P.; Lu, H.; Makara, GM.; Romero, FA.; Sebat, I.; Wilson, D.; Wodka, D. Merck Sharp & Dohme Corp. USA: Metabasis Therapeutics, Inc.; 2010. p. 149
22. Xiao B, Sanders MJ, Carmena D, Bright NJ, Haire LF, Underwood E, Patel BR, Heath RB, Walker PA, Hallen S, Giordanetto F, Martin SR, Carling D, Gamblin SJ. Nat Commun. 2013; 4:3017. [PubMed: 24352254]
23. Lima LM, Barreiro EJ. Curr Med Chem. 2005; 12:23–49. [PubMed: 15638729]
24. Meanwell NA. J Med Chem. 2011; 54:2529–2591. [PubMed: 21413808]
25. Dowling RJ, Zakikhani M, Fantus IG, Pollak M, Sonenberg N. Cancer Res. 2007; 67:10804–10812. [PubMed: 18006825]
26. Baell JB, Ferrins L, Falk H, Nikolakopoulos G. Aust. J. Chem. 2013; 66:1483–1494.
27. Baell JB, Holloway GA. J Med Chem. 2010; 53:2719–2740. [PubMed: 20131845]

28. Baell J, Walters MA. Nature. 2014; 513:481–483. [PubMed: 25254460]
29. Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC, Ferrin TE. J Comput Chem. 2004; 25:1605–1612. [PubMed: 15264254]

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 1.

OSU-53 (**1**) as a scaffold to develop potent AMPK activators. (A) Docking of **1** into the allosteric site formed at the interface between the kinase domain and CBM of AMPK enzyme (PDB ID, 4CFE). (B) A schematic diagram depicting strategies for the lead optimization of **1**. (C) General synthetic procedures for **2** – **87**. Reaction conditions: (a) ArCHO, toluene, AcOH, piperidine, 80°C, overnight; (b) ArCH₂Br, MeCN, K₂CO₃, r.t., 2 days; (c) SnCl₂·2H₂O, EtOAc, reflux, 4-8 h; (d) ArSO₂Cl, pyridine, r.t., 3 days; (e) Derivatives **2** or **3**, BH₃·DMS, THF, 70°C, overnight; (f) NaN₃, NH₄Cl, DMF, 125°C, 24 h; (g) Morpholine carbonyl chloride, pyridine, r.t., overnight; (h) ArCOCl, pyridine, r.t., overnight; (i) Phosgene, toluene, 100°C, 2 h; (j) ArNH₂, toluene, 100°C, 12 h; (k) ArNCS, DMF, r.t., 12 h; (l) 10% Pd/C, MeOH, 60 psi, 16 h.

Figure 2.
Structures of **2** – **86** in Series **A** – **G**.

Figure 3.

Identification of optimal AMPK activators. (A) Effects of **1** – **86**, each at 5 μ M, relative to DMSO control on the viability of MDA-MB-231 cells after 72 h of incubation by MTT assays. Columns, mean; bars, S.D. (n = 6). *, compounds with the ability to suppress $\geq$ 75% cell viability. Western blot analysis of (B) dose-dependent effects of metformin (left) and AICAR (right), and (C) effects of selected derivatives, each at 2.5 μ M, on AMPK phosphorylation in MDA-MB-231 cells after 48 h of treatment.

Figure 4.

(A) Time [24 h (square), 48 h (triangle) and 72 h (circle)] and dose-dependent suppressive effects of **51**, **59**, and **71** versus **1** on the viability of MDA-MB-231 cells, as determined by MTT assays. Data are presented as means $\pm$ S.D. (n = 6). (B) Western blot analysis of the dose-dependent effects of individual agents on the phosphorylation of AMPK and downstream targets, including ACC, p70S6K, and Akt in MDA-MB-231 cells after 48 h of exposure. (C) Dose-dependent effects of **60** on the phosphorylation of AMPK in MDA-MB-231 cells after 48 h of exposure.

Figure 5.

Western blot analysis of the effect of compound **59** versus **1** on the phosphorylation of AMPK, ACC, p70S6K, and Akt in (A) MDA-MB468 and (B) PC-3 cells (B) after 48 h of treatment. (C) Suppressive effect of **59** on EMT in MDA-MB-231 and PC-3 cells, as manifested by increases in the expression of Foxo3a and the epithelial marker claudin-1 or E-cadherin, accompanied by parallel decreases in the expression of the mesenchymal markers vimentin and snail.

Figure 6. Evidence that **59** is not a PAINS compound. (A) Structure of **87**, a saturated derivative of **59** (left), Western blot analysis of the dose-dependent effects of **87** on the phosphorylation of AMPK in MDA-MB-231 cells after 48 h of exposure (right). (B) Time dependent effects of **59**, via continuous exposure or removal (washout) after 12 h of drug exposure, on the phosphorylation of AMPK in MDA-MB-231 cells.

Figure 7.

(A) *In vivo* efficacy of **59** (circle) versus **1** (triangle) in suppressing PC-3 xenograft tumor growth. Vehicle control (square) is shown for comparison. Athymic nude mice bearing established subcutaneous PC-3 xenograft tumors were treated once daily with vehicle, **1**, or **59** via oral gavage at 50 mg/kg for 28 days. Tumor volume (left) and body weight (right) data are presented as means $\pm$ S.E. ($n = 7$). (B) Western blot analysis of the phosphorylation

of AMPK, ACC, and Akt in lysates from four representative tumors from each group of mice after 28 days of treatment.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript