Brief Communication

Chemistry & Biology

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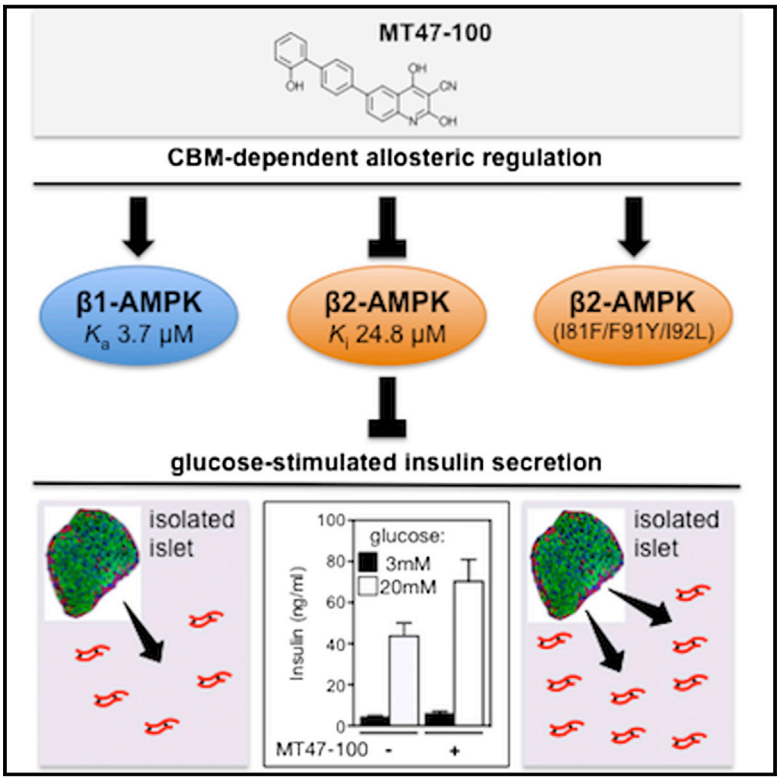
Graphical Abstract



Highlights

- MT47-100 is an allosteric inhibitor of AMPK complexes containing the β2 isoform
- β2-CBM, but not Ser108 phosphorylation, is required for AMPK inhibition
- Isoform specificity of MT47-100 is determined by three β2 residues
- MT47-100 augmented glucose-stimulated insulin secretion from isolated islets

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In Brief

AMP-activated protein kinase (AMPK) is a central regulator of energy metabolism. Therapeutic AMPK inhibition is regarded as a strategy to combat diabetes, cancers, and neurodegeneration. Scott et al. have identified MT47-100 as an AMPK inhibitor acting through an allosteric drug-binding site. These findings will aid development of AMPK-targeting therapeutics.

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Chemistry & Biology
Brief Communication

Inhibition of AMP-Activated Protein Kinase at the Allosteric Drug-Binding Site Promotes Islet Insulin Release

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SUMMARY

The AMP-activated protein kinase (AMPK) is a metabolic stress-sensing αβγ heterotrimer responsible for energy homeostasis. Pharmacological inhibition of AMPK is regarded as a therapeutic strategy in some disease settings including obesity and cancer; however, the broadly used direct AMPK inhibitor compound C suffers from poor selectivity. We have discovered a dihydroxyquinoline drug (MT47-100) with novel AMPK regulatory properties, being simultaneously a direct activator and inhibitor of AMPK complexes containing the β1 or β2 isoform, respectively. Allosteric inhibition by MT47-100 was dependent on the β2 carbohydrate-binding module (CBM) and determined by three non-conserved CBM residues (Ile81, Phe91, Ile92), but was independent of β2-Ser108 phosphorylation. Whereas MT47-100 regulation of total cellular AMPK activity was determined by β1/β2 expression ratio, MT47-100 augmented glucose-stimulated insulin secretion from isolated mouse pancreatic islets via a β2-dependent mechanism. Our findings highlight the therapeutic potential of isoform-specific AMPK allosteric inhibitors.

INTRODUCTION

The AMP-activated protein kinase (AMPK) is an important regulator of cellular and whole-body energy balance that synchronizes metabolic processes to match energy supply with demand (Hardie, 2007; Steinberg and Kemp, 2009). AMPK directly monitors adenylate nucleotide ratios and protects the cell from events that perturb energy charge (nutrient deprivation, hypoxia, exercise) by directing cellular metabolism away from anabolic processes toward catabolic pathways. It achieves this acutely by direct phosphorylation of rate-limiting enzymes in major metabolic pathways, and chronically by phosphorylation of transcription factors that control expression of metabolic genes. AMPK also integrates an array of hormonal and nutritional signals in the CNS and periphery to control appetite and body weight (Kahn et al., 2005; Steinberg and Kemp, 2009).

The AMPK αβγ heterotrimer consists of an α catalytic subunit and regulatory β and γ subunits that contain a carbohydrate-binding module (CBM) and allosteric adenylate nucleotide-binding sites, respectively (Oakhill et al., 2009). Multiple isoforms of each subunit exist in mammals (α1/2, β1/2, γ1/2/3), and isoform-specific variations in tissue distribution, regulation, and function have been demonstrated (Steinberg and Kemp, 2009). AMPK signaling is initiated by phosphorylation of Thr172 in the α-subunit activation loop by upstream kinases LKB1 or Ca^{2+}/calmodulin-dependent protein kinase kinase β (CaMKKβ). LKB1-mediated phosphorylation is stimulated by AMP (Oakhill et al., 2010), but whether AMP and ADP also stimulate CaMKKβ-mediated phosphorylation remains controversial (Oakhill et al., 2011; Gowans et al., 2013). AMP allosterically activates phosphorylated AMPK, and both AMP and ADP maintain the active state by suppressing dephosphorylation of phosphorylated Thr172 (Oakhill et al., 2012; Gowans et al., 2013). AMP also synergizes with the β1-specific activating drug A-769662 (Cool et al., 2006; Scott et al., 2008) to substantially activate unphosphorylated AMPK (Scott et al., 2014). The A-769662-binding site is stabilized by phosphorylation of β-CBM Ser108 and is located at the interface of the α-kinase domain small lobe and β-CBM, as recently revealed by crystal structures of AMPK drug complexes (Xiao et al., 2013; Calabrese et al., 2014).

Whereas the beneficial effects of AMPK activators are well documented, the development of AMPK inhibitors is lacking even though AMPK inhibition plays an important role in whole-body energy homeostasis (Viollet et al., 2010). The first

AMPK inhibitor described was compound C (Zhou et al., 2001), which competes with ATP at the highly conserved kinase active site (Handa et al., 2011) and displays widely accepted off-target effects. Here, we describe an A-769662-related compound, MT47-100, which acts uniquely as a β2-specific allosteric AMPK inhibitor. This study demonstrates the potential for developing small-molecule inhibitors with improved selectivity for AMPK.

RESULTS

AMPK β Isoforms Mediate Opposing Allosteric Effects of MT47-100

In the course of investigating small-molecule AMPK regulators, we found that the compound MT47-100 was a direct activator of AMPK complexes containing the β1-isoform ($AMPK_{\beta1}$) in cell-free assay (Figures 1A and 1B) (Mercury Therapeutics Inc. patent WO2009/100130 A1). MT47-100 is structurally similar to A-769662 but possesses a dihydroxyquinoline core instead of the thienopyridone core of A-769662. MT47-100 maximally activated α1β1γ1 approximately 2.5-fold with half-maximal activation K_a = 3.7 ± 0.5 μM, compared with 6.7 ± 0.6 μM for AMP (Figure 1C), and activated all $AMPK_{\beta1}$ complexes regardless of α or γ isoform (Figure 1B). Like A-769662, $AMPK_{\beta1}$ sensitivity to MT47-100 activation was lost following deletion of the β1 subunit N-terminal 145 residues including the CBM (Figure 1D) and substitution of the phosphorylated residue β-Ser108 to Ala, but retained following exchange of Ser108 for the phosphomimetic Glu (Figure 1E). MT47-100 also synergistically activated dephosphorylated $AMPK_{\beta1}$ in the presence of AMP (Figure S1).

In contrast, MT47-100 directly inhibited $AMPK_{\beta2}$ complexes independently of α or γ isoform (Figure 1B). Approximately 93% inhibition of α1β2γ1 activity was obtained following incubation with 200 μM MT47-100, with half-maximal inhibition K_i = 24.8 ± 2.8 μM (Figure 1F). Truncation of the β2 subunit N-terminal 145 residues containing the CBM resulted in loss of MT47-100 inhibition (Figure 1G), but mutation of β2-Ser108 to Ala did not diminish sensitivity to MT47-100 (Figure 1H). These data demonstrate that MT47-100 both activates $AMPK_{\beta1}$ and inhibits $AMPK_{\beta2}$ through a CBM allosteric site.

Three β-CBM Residues Mediate Isoform-Specific Allosteric Properties of MT47-100

To identify residues that mediate agonistic/antagonistic properties of MT47-100, we expressed AMPK containing β-subunit chimeras (Figure 2A). Replacement of the C-terminal 158 residues with the corresponding sequence from the alternative β isoform (to yield $AMPK_{\beta1\text{-}2}$ or $AMPK_{\beta2\text{-}1}$) had no significant effect on regulation by MT47-100 (Figure 2B). MT47-100 also activated an $AMPK_{\beta1\text{-}2\text{-}1}$ chimera in which β1 residues 100–112 were substituted with the corresponding β2 sequence, indicating the determinants of drug specificity were localized to the N-terminal 100 residues (Figures 2A and 2B).

We next generated individual and combinatorial mutants in which the eight non-conserved β1 CBM residues between positions 73 and 99 were exchanged for the corresponding β2 residue (Figure S2A). Double substitution of β1-Phe82 and β1-Leu93 to Ile (β1(F82I/L93I)) was sufficient to reverse the stimulatory effect of MT47-100, as this mutant was significantly inhibited (32%) by MT47-100 (Figure 2C). The activity of a triple mutant containing the additional substitution Y92F (β1(F82I/Y92F/L93I) was further suppressed by MT47-100 (79% inhibition) (Figure 2C). The reciprocal β2 to β1 substitutions (β2(I81F/F91Y/I92L)) rendered $AMPK_{\beta2}$ sensitive to activation by MT47-100 (Figure 2D). In addition, the double mutant I81F/I92L was sufficient to sensitize $AMPK_{\beta2}$ to allosteric activation by A-769662 (Figure 2E). Thus β-isoform selectivity displayed by direct AMPK activators/inhibitors can be mapped to three CBM residues: Phe82, Tyr92, and Leu93 in β1 (Figure S2B) and the corresponding residues Ile81, Phe91, and Ile92 in β2.

Regulation of AMPK Signaling by MT47-100

To determine whether the β-isoform specificity of MT47-100 displayed in vitro was reflected in cellular regulation of AMPK signaling, we first investigated β-isoform expression profiles in cultured cell lines. AMPK β1 subunit displays reduced mobility on SDS-PAGE, whereas β2 migration is in accordance with theoretical mass. Using N-terminal truncations, we determined that reduced electrophoretic mobility of β1 is largely due to a non-conserved sequence between residues 40 and 60 (Figure S3A), which contains a region of predicted α-helical secondary structure (Figure S3B). $AMPK_{\beta1}$ content was highest in HepG2 cells (93% β1), whereas HEK293 cells possessed the highest proportion of $AMPK_{\beta2}$ (58% β2) (Figure S3C). MT47-100 exposure resulted in dose-dependent increases in phosphorylation of the AMPK substrate acetyl-CoA carboxylase 1 (ACC1) in hepatocytes derived from wild-type (WT) mice, but not mice with constitutive β1-subunit deletion (β1 knockout [KO]) (Figure 3A). We attribute these effects to β1-specific activation, since MT47-100 similarly increased ACC phosphorylation (pACC) in HepG2 cells without significantly increasing the AMP/ATP ratio at concentrations below 400 μM (Figures S3D and S3E). Consistent with its capacity to positively regulate $AMPK_{\beta1}$, MT47-100 significantly inhibited diacylglycerol (Figure 3B) and triacylglycerol (Figure 3C) synthesis in WT, but not β1KO, hepatocytes.

To assess MT47-100 inhibition of AMPK signaling, we used hepatocytes derived from AMPK β1KO mice, which we previously demonstrated exclusively express $AMPK_{\beta2}$ (Scott et al., 2008). Basal levels of pACC in these hepatocytes were undetectable (Figure 3D); therefore, we examined the effect of MT47-100 on cells treated with the AMPK-activating agent AICAR. Hepatocytes from β1KO mice displayed an increase in AICAR-induced pACC (Figure 3D) similar to that in WT, indicating that $AMPK_{\beta2}$ in these cells was able to fully compensate for loss of $AMPK_{\beta1}$. Pretreatment with MT47-100 (≥200 μM) abolished the response in β1KO hepatocytes only (Figure 3D). We previously implicated $AMPK_{\beta2}$ signaling in mediating AICAR-stimulated glucose uptake in mouse skeletal muscle (Steinberg et al., 2010). Consistent with this finding, MT47-100 significantly suppressed both basal and AICAR-stimulated glucose uptake in extensor digitorum longus (EDL) muscle isolated from WT, but not β2KO, mice (Figure 3E). Taken together, our results confirm that MT47-100 inhibits $AMPK_{\beta2}$-dependent signaling in cells and isolated tissue.

MT47-100 Augments Glucose-Stimulated Insulin Secretion from Isolated Islets via an $AMPK_{\beta2}$-Dependent Mechanism

We investigated the effect of MT47-100 on glucose-stimulated insulin secretion (GSIS) from pancreatic β cells, which represents

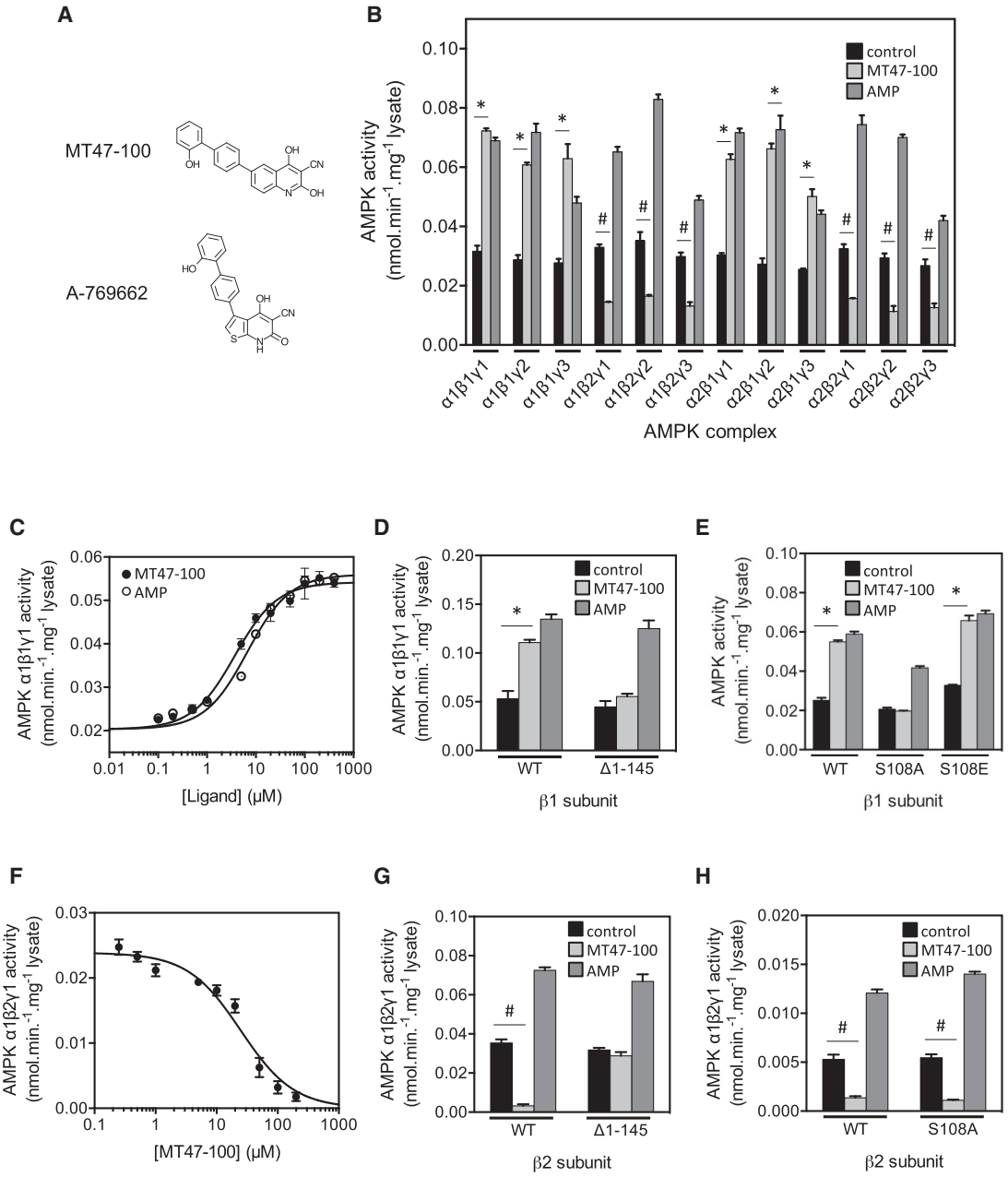

Figure 1. Biochemical Characterization of MT47-100

Data are presented as means ± SEM (n = 3–4). Significant differences between basal and MT47-100 incubated activities are shown (*$p < 0.01$ for activation, #$p < 0.01$ for inhibition).

(A) Structures of AMPK regulators MT47-100 (upper) and A-769662 (lower).

(B) MT47-100 directly activates $AMPK_{\beta1}$ and directly inhibits $AMPK_{\beta2}$. Activities of purified AMPK complexes were measured by SAMS assay ± MT47-100 (20 μM) or AMP (100 μM).

(C) Dose responses for MT47-100 and AMP allosteric activation of α1β1γ1.

(D) MT47-100 activation of $AMPK_{\beta1}$ is CBM dependent. Activities of AMPK containing wild-type (WT) or CBM-truncated (Δ1–145) β1 subunit were measured by SAMS assay ± MT47-100 (100 μM) or AMP (100 μM).

(E) MT47-100 activation of $AMPK_{\beta1}$ is dependent on phosphorylation of β-Ser108. AMPK (WT and β1-Ser108 mutants as indicated) activity was measured following incubation with MT47-100 (20 μM) or AMP (100 μM).

(F) Dose response for MT47-100 allosteric inhibition of α1β2γ1.

(G) MT47-100 inhibition of $AMPK_{\beta2}$ is CBM dependent. Activities of AMPK containing WT or CBM-truncated (Δ1–145) β2 subunit were measured by SAMS assay ± MT47-100 (20 μM) or AMP (100 μM).

(H) MT47-100 inhibition of $AMPK_{\beta2}$ is independent of Ser108 phosphorylation. Activities of AMPK containing WT or S108A mutated β2 subunit were measured by SAMS assay ± MT47-100 (100 μM) or AMP (100 μM).

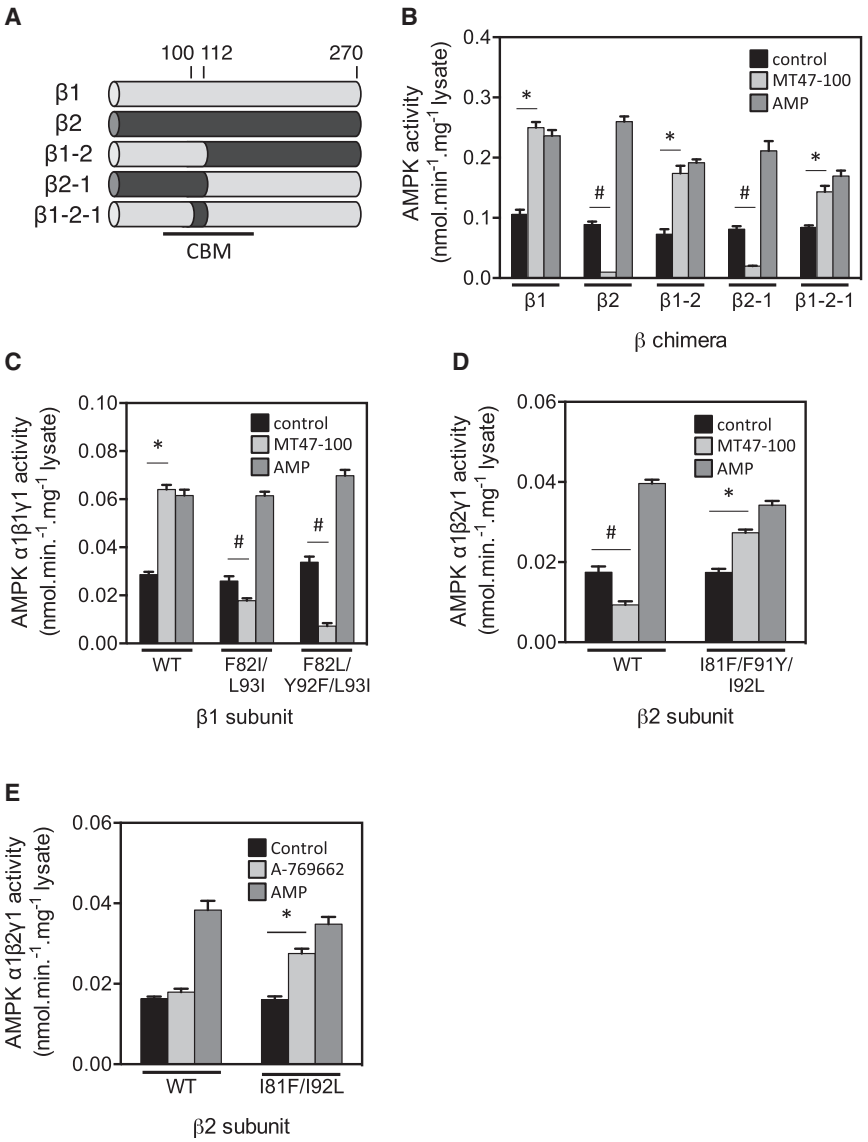

Figure 2. Identification of AMPK β Residues Mediating Isoform-Specific Allosteric Properties of MT47-100

Data are presented as means ±SEM (n = 4). Significant differences between basal and MT47-100/A-769662 incubated activities are shown (*$p < 0.01$ for activation, #$p < 0.01$ for inhibition).

(A and B) Activities of purified AMPK containing β chimeras (A) were measured by SAMS assay (B) ± MT47-100 (20 μM) or AMP (100 μM).

(C–E) Activities of AMPK containing WT or mutant β1 or β2 as indicated were measured by SAMS assay (C and D) ± MT47–100 (20 μM) or AMP (100 μM), or (E) ± A-769662 (10 μM) or AMP (100 μM).

a potential target for therapeutic inhibition of AMPK (Rutter and Leclerc, 2009). The relative expression level of $AMPK_{\beta1}$ and $AMPK_{\beta2}$ in mouse islets was measured at 71% and 29%, respectively, resembling that in human islets (Figure 4A). Despite this lower relative $AMPK_{\beta2}$ abundance, serum insulin in the fasted state was significantly elevated in constitutive β2KO mice compared with WT (Figure 4B). Our findings are consistent with a negative regulatory role for $AMPK_{\beta2}$ in insulin secretion (da Silva Xavier et al., 2000), but contrast with suppressed insulin release observed in mice with pancreatic β-cell-/RIP.Cre2 neuron-specific deletion of both α1 and α2 isoforms (Sun et al., 2010). GSIS from isolated mouse islets was increased >1.6-fold with prior MT47-100 incubation (Figure 4C). To confirm AMPK dependence, we measured GSIS from islets extracted from β1KO or β2KO mice. Islets derived from either KO mouse line displayed normal glucose-stimulated responses in the absence of MT47-100. Whereas the MT47-100-mediated increase in glucose responsiveness was retained in β1KO islets, the augmented response was entirely lost in islets from β2KO mice (Figure 4C).

DISCUSSION

A range of nutrient, hormonal, and cytokine signals contribute to energy homeostasis through AMPK inhibition (Viollet et al., 2010), and pharmacological inhibition of AMPK is considered to confer benefit in a range of disease settings, e.g. cancer (suppression of tumor growth), stroke (neuroprotection), obesity (appetite regulation), and, as recently demonstrated, Alzheimer's disease (Ma et al., 2014). In addition, AMPK inhibition in pancreatic β cells in response to elevated serum glucose is regarded as a requirement for upregulation of insulin secretion (Rutter and Leclerc, 2009). We view MT47-100 as the founding member of a novel class of compounds that act as AMPK allosteric inhibitors. This represents a major advance in the development of AMPK inhibitors with potentially higher selectivity than compound C, an agent used in more than 750 published studies. Compound C competes with ATP at the highly conserved active site, and suffers from selectivity concerns since it was shown to potently inhibit a range of kinases in vitro (Bain et al., 2007; updated at http://www.kinase-screen.mrc.ac.uk/screening-compounds/341053). Our data indicate that MT47-100 inhibition of $AMPK_{\beta2}$ is mediated through an alternative CBM-dependent site.

Whereas MT47-100 likely binds to the recently identified drug site in $AMPK_{\beta1}$ to effect activation, we cannot formally rule out the possibility that the inhibitory site in $AMPK_{\beta2}$ is distinct from the activator site. However, it seems reasonable that MT47-100 occupies essentially the same site in $AMPK_{\beta2}$ as the β1 agonists do in $AMPK_{\beta1}$, but in such a way as to promote an inhibited form of the enzyme. This interpretation is consistent with the mapping of key residues responsible for switching agonist/antagonist specificity for $AMPK_{\beta1}$ (Phe82, Tyr92, Leu93) and $AMPK_{\beta2}$ (Ile81, Phe91, Ile92). While these residues do not directly contribute to the drug-binding pocket, they stack,

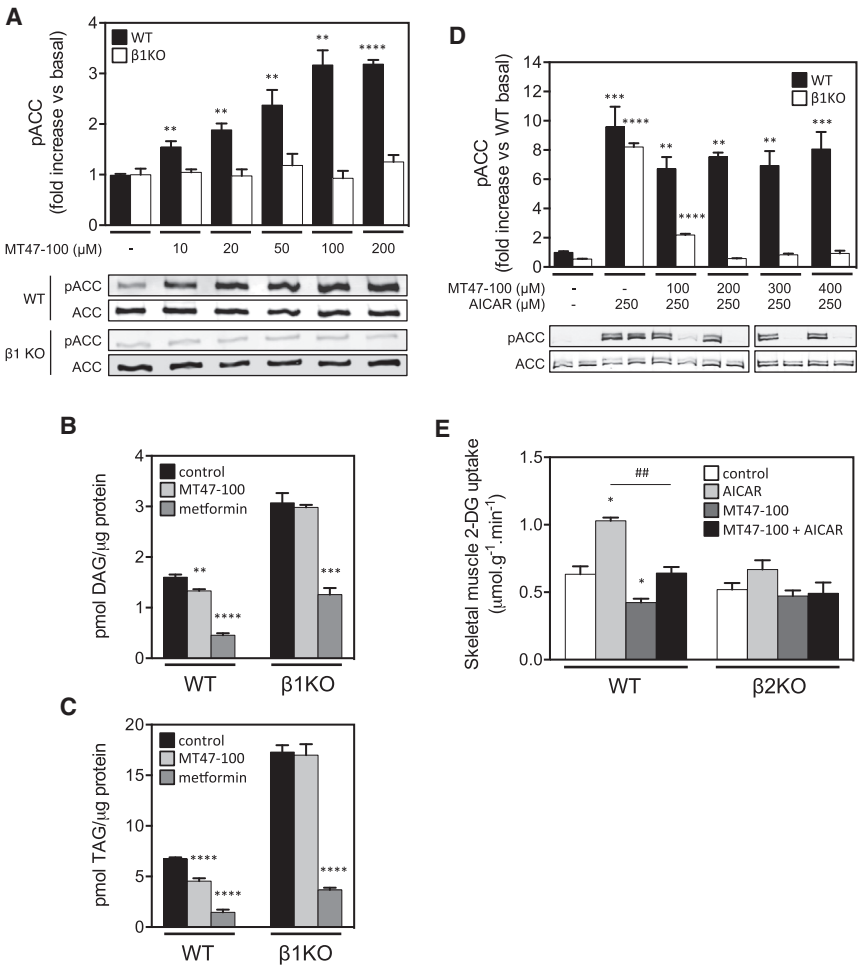

Figure 3. MT47-100 Regulation of Cellular AMPK Signaling

Data are presented as means ± SEM.

(A) MT47-100 promotes ACC1 phosphorylation in WT, but not β1KO, hepatocytes. Hepatocytes were harvested after 1 hr of incubation with indicated concentrations of MT47-100 and lysates immunoblotted for ACC1-pSer79. Significant differences in ACC1 phosphorylation between untreated and MT47-100 incubated cells are shown (n = 3; **p < 0.01, ****p < 0.0001).

(B) Diacylglycerol (DAG) and (C) triacylglycerol (TAG) synthesis is inhibited by MT47-100 in WT, but not β1KO, hepatocytes. Hepatocytes were harvested after 1 hr of incubation with 100 μM MT47-100 or 1 mM metformin as a positive control. Significant differences in DAG or TAG synthesis between untreated and incubated cells are shown (n = 3–7; **p < 0.01, ***p < 0.001, ****p < 0.0001).

(D) MT47-100 attenuates AICAR-induced ACC phosphorylation in β1KO hepatocytes. Primary hepatocytes isolated from WT or β1KO mice were pre-incubated for 1 hr with indicated concentrations of MT47-100, prior to 30 min of incubation with AICAR (250 μM). Harvested lysates were immunoblotted for ACC phosphorylation. Statistical analyses were performed by one-way ANOVA using Dunnett's multiple comparison test. Significant differences in ACC phosphorylation between untreated and AICAR/MT47-100 incubated hepatocytes from each mouse line are shown (n = 3; **p < 0.01, ***p < 0.001, ****p < 0.0001).

(E) MT47-100 attenuation of AICAR-induced 2-[^{14}C] deoxyglucose (2-DG) uptake in skeletal muscle ex vivo is dependent on AMPK$_{\beta2}$-signaling. 2-DG uptake was measured in EDL muscle isolated from WT or β2KO mice. Muscles were pre-incubated for 30 min with DMSO or 80 μM MT47-100. Muscles pre-incubated with DMSO were incubated for a further 30 min with either DMSO (control) or 150 μM AICAR (AICAR). Muscles pre-incubated with MT47-100 were incubated for a further 30 min with either 80 μM MT47-100 (MT47-100) or 80 μM MT47-100 + 150 μM AICAR (MT47-100 + AICAR). Statistical analyses were performed by non-parametric permutation test. Significant differences in 2-DG uptake between treatments are shown (n = 5–7; *p < 0.05 versus untreated, ##p < 0.01 versus AICAR-treated).

together with the drug-contacting residue β1-Arg83, to form a "spine" through the CBM core (Figure S2B). This connectivity may be a key to their importance.

Our observation that MT47-100-induced augmentation of GSIS from mouse islets occurs via AMPK$_{\beta2}$-specific signaling is in accordance with a previous study showing that inhibition of AMPK$_{\beta2}$ in MIN6 cells by antibody microinjection stimulated activity of the insulin promoter (da Silva Xavier et al., 2000). MT47-100 would likely produce a similar response in human islets given that AMPK β-isoform distribution is comparable with that of the mouse. Therapeutic use of MT47-100 is limited by low potency; however, our study provides further proof of concept regarding the potential health benefits of small compound AMPK inhibitors.

SIGNIFICANCE

As a central mediator of energy homeostasis AMPK is subject to tight regulatory control, yet thus far most emphasis has been placed on investigating agonistic mechanisms rather than antagonistic ones. We have demonstrated that drugs can act as allosteric inhibitors of AMPK. Our findings provide a lead in the development of small compounds to study the role of AMPK inhibition in multiple diseases including obesity, cancer, Alzheimer's disease, and, as described here, diabetes. In addition, the β-isoform-specific regulatory properties of MT47-100 highlight the importance in AMPK drug-screening strategies to employ the full range of AMPK isoforms.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies

MT47-100 was from CreaGen and A-769662 was from Tocris. Antibodies were from Cell Signaling (pan AMPK α [#2793], AMPK α-pThr712 [#2535], pan AMPK β [#4150], AMPK β1-pSer108 [#4181], ACC1-pSer79 [#3661]). IRDye 680RD/800CW labeled anti-immunoglobulin G antibodies and streptavidin were from LI-COR Biosciences.

AMPK Production and Activity Assay

All mutants were generated using QuikChange site-directed mutagenesis kits (Stratagene), and β chimeras were generated by two-step PCR. All constructs

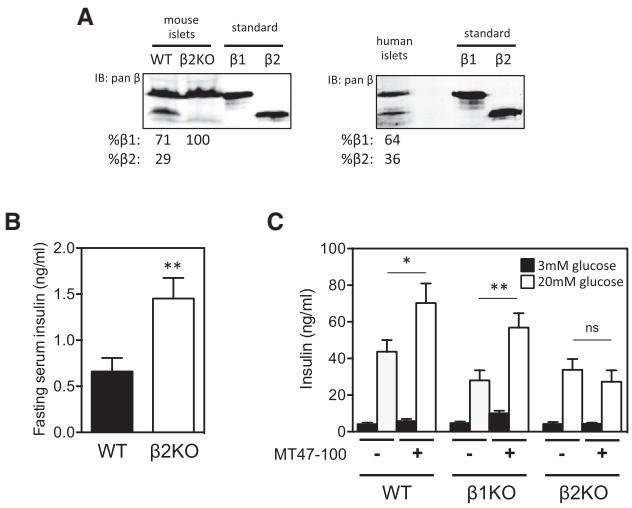

Figure 4. MT47-100 Promotes Glucose-Stimulated Insulin Secretion in Isolated Mouse Islets

Data are presented as means ± SEM.

(A) AMPK β-isoform content of mouse (WT and β2KO, left) and human (right) islets. Isolated islets (mouse: 700; human: 300) were immunoblotted with pan AMPK β antibody against purified, *E. coli*-expressed $AMPK_{β1}$ and $AMPK_{β2}$ standards. Values below indicate expression of each β isoform as a percentage of total β.

(B) AMPK β2 deletion is associated with hyperinsulinemia after a 16-hr fast (n = 16–18, combined from two independent experiments; **p < 0.01 versus WT).

(C) Insulin secretion assays were performed on untreated or MT47-100 (200 μM) incubated islets isolated from C57Bl/6 WT, β1KO, or β2KO mice as indicated. Insulin secretion into the supernatant was analyzed by mouse insulin ELISA. Significant differences in insulin secretion at 20 mM glucose between untreated islets and islets incubated with MT47-100 are shown (n = 6–13, combined from five independent experiments; *p < 0.05, **p < 0.01; ns, not significant).

were sequence verified. Heterotrimeric human AMPK (GST-α1/β1-Myc/HA-γ1 and GST-α1/β2-Myc/HA-γ1; WT and mutants, chimeras, and truncations as indicated) were expressed in COS7 cells as previously described (Oakhill et al., 2010). AMPK was isolated from COS7 cell lysates on anti-c-Myc affinity gel (Sigma) prior to assay. AMPK for AMP/MT47-100 synergy analysis was isolated on glutathione Sepharose 4B (GE Life Sciences), dephosphorylated with λ-phosphatase (2 mM $MnCl_2$, 2 hr, 22°C), extensively washed with 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 10% glycerol, and 1 mM DTT (buffer A), and eluted with buffer A supplemented with 20 mM glutathione prior to assay. All AMPK preparations were quantitated as described previously (Scott et al., 2014). AMPK activity was determined by phosphorylation of the SAMS peptide as previously described (Scott et al., 2014).

Insulin Secretion Assay

GSIS assays were performed as previously described (Thomas et al., 2002).

Ethics Statement

Human pancreata were obtained, with informed consent from next-of-kin, from heart-beating, brain-dead donors by the Australian Islet Transplant Consortium. The human ethics committees of the hospitals involved and the Australian Red Cross approved the project.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and three figures and can be found with this article online at http://dx.doi.org/10.1016/j.chembiol.2015.05.011.

AUTHOR CONTRIBUTIONS

J.W.S., S.G., B.E.K., and J.S.O. designed the study. J.W.S., S.G., and K.L.G. performed the experiments. N.X.Y.L. and C.G.L. prepared reagents. R.F., Q.P.W., and N.C.B. prepared MT47-100. T.A.D. and S.M.A.I. performed nucleotide measurements. G.R.S., H.E.T., and T.W.K. provided conceptual input. J.S.O., J.W.S., and B.E.K. wrote the manuscript.

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