HHS Public Access

Author manuscript

J Med Chem. Author manuscript; available in PMC 2016 May 12.

Published in final edited form as:

J Med Chem. 2015 January 8; 58(1): 2–29. doi:10.1021/jm401994c.

Small molecule adenosine 5′-monophosphate activated protein kinase (AMPK) modulators and human diseases

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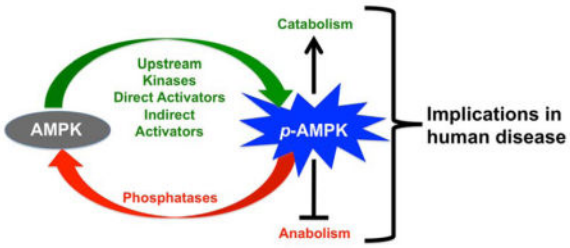
Abstract

Adenosine 5′-monophosphate activated protein kinase (AMPK) is a master sensor of cellular energy status that plays a key role in the regulation of whole-body energy homeostasis. AMPK is a serine/threonine kinase that is activated by upstream kinases LKB1, CaMKKβ and Tak1 among others. AMPK exists as αβγ trimeric complexes that are allosterically regulated by AMP, ADP and ATP. Dysregulation of AMPK has been implicated in a number of metabolic diseases including type 2 diabetes mellitus and obesity. Recent studies have associated roles of AMPK with the development of cancer and neurological conditions making it a potential therapeutic target to treat human diseases. This perspective focuses on the structure and function of AMPK, its role in human diseases and its direct substrates and provides a brief synopsis of key AMPK modulators and their relevance in human diseases.

Graphical Abstract



Introduction

AMP-activated protein kinase (AMPK), which is present in all eukaryotes, is a master sensor of metabolic stress and exists as heterotrimeric αβγ complexes. AMPK is a nutrient and energy sensor that plays a key role in whole-body energy homeostasis.[1,2] Its cellular functions are heavily dependent on ATP levels and alterations in the cellular AMP : ADP : ATP ratio lead to the activation or deactivation of AMPK. In response to energy needs (*i.e.*, reduced ATP levels or increased AMP levels), AMPK is activated. Activated AMPK

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Notes

The authors declare no competing financial interest.

phosphorylates a plethora of substrates in metabolic pathways resulting in the inhibition of anabolic pathways and the activation of catabolic pathways.[3–19]

AMPK plays a central role in maintaining the energy and metabolic landscape of cells. An altered metabolic profile is often used as a biomarker in chronic human conditions such as diabetes, Alzheimer's disease (AD), and cancer, and AMPK is implicated in these alterations. For example, AD is characterized by the accumulation of Amyloid-beta protein (Aβ). In cerebrospinal fluid, $A\beta_{1-42}$ was identified as a potential biomarker for Alzheimer's disease.[20] Aβ peptide generation is increased in AMPKα2 knockout neurons and decreased in the presence of the AMPK stimulator 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) suggesting AMPK is a key regulator of Aβ accumulation.[21] AMPK dysfunction leads to altered cholesterol and sphingomyelin levels, which changes the distribution of amyloid precursor protein (APP), the source of Aβ, in lipid rafts. This is the current and well-accepted model for the role of AMPK in the accumulation of Aβ.[21] This suggests that activation of AMPK by small molecules may be a viable therapeutic approach for restoring the energy and metabolic landscape and reversing the disease phenotype.

Regulation of AMPK by adenine nucleotides

AMPK is a heterotrimeric kinase, composed of a highly conserved catalytic α subunit and a regulatory β and γ subunit. The catalytic α subunit and the regulatory β subunit exist as two isoforms (α1, α2 and β1, β2) respectively. The regulatory γ subunit exists as three isoforms (γ1, γ2 and γ3). These seven gene products lead to 12 possible heterotrimeric combinations: α1β1γ1, α1β1γ2, α1β1γ3, α1β2γ1, α1β2γ2, α1β2γ3, α2β1γ1, α2β1γ2, α2β1γ3, α2β2γ1, α2β2γ2, α2β2γ3. The domain architecture of the α1, β2 and γ1 subunits is summarized in Figure 1. The α subunit is composed of a serine/threonine kinase domain (KD), an autoinhibitory domain (AID), a α-hook domain and a C-terminal β subunit-binding domain. The β subunit is composed of a glycogen binding domain (GBD) and a C-terminal domain that has binding sites for α and γ subunits. The γ subunit has a β subunit-binding region and two Bateman domains that are assembled in a head-to-head manner. The Bateman domains are composed of two tandem cystathionine β-synthase (CBS) motifs.

The kinase domain of the α subunit is activated upon phosphorylation of Thr-172 of the activation loop.[22] Upstream kinases, such as liver kinase B1 (LKB1)[23,24] calcium/calmodulin-dependent protein kinase kinase β (CaMKKβ),[25,26] and mammalian transforming growth factor-β activated protein kinase-1 (TAK1),[27] phosphorylate Thr-172. Phosphorylation of Thr-172 leads to a 2–3 orders of magnitude increase in AMPK activity.[28] Deactivation of AMPK occurs through dephosphorylation of Thr-172.[28,29] Studies by Voss *et al.* identified Mg^{2+}/Mn^{2+}-dependent protein serine/threonine phosphatase (Ppm) 1E as an AMPK phosphatase. Briefly, in HEK293 cells, depletion of Ppm1E by RNAi strategies increased Thr-172 phosphorylation.[30] Allosteric effects such as binding of adenine nucleotides to the γ-domain, which will be discussed shortly, regulate the conformations around Thr-172 to allow or deny access to upstream kinases and phosphatases.

A truncated α subunit lacking the AID showed full kinase activity when compared to a α subunit containing both the KD and AID. Structural studies with the α subunit of AMPK

from *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* reveal that hydrophobic residues drive the KD-AID interaction. Movement of the helix α-C in the KD is probably constrained upon AID binding, thus forcing the KD into a relatively open conformation. Point mutations of the hydrophobic residues in the AID to charged residues (L341D, L342D and M316E) increased the kinase activity by ten-fold. These studies support the regulation of the KD conformation by AID binding to the hydrophobic patch on the KD.[31]

The heterotrimeric AMPK complex is held together by the β subunit. It has a Cterminal α-subunit binding domain, which terminates in a short peptide sequence that interacts with the β subunit-binding domain of the γ subunit. The N-terminus of the β subunit is modified by myristoylation, which is suggested to facilitate shuttling of the AMPK complex between the cytoplasm and the nucleus.[32] The GBD in the β subunit of AMPK is similar to carbohydrate-binding modules (CBM) found in proteins that are known to metabolize starch and glycogen. Glycogen particles are in complex with glycogen synthase (GS) and the GBD on the β subunit helps the AMPK complex to bind to the surface of glycogen particles.[33] Isoforms of GS found in the liver and muscle are known substrates of AMPK and phosphorylation of GS could inhibit the anabolic process of glucose addition to glycogen.

There are four CBS motifs present in γ subunit and three out of four CBS motifs recognize and bind adenine nucleotides (Figure 2).[34] Adenine nucleotide binding SITE-1 and SITE-3 on the γ subunit lie on opposite faces and can exchangeably bind AMP, ADP or ATP with SITE-1 having a higher affinity for all three nucleotides than SITE-3. In Figure 2 we show ATP binding to SITE-1 and SITE-3. SITE-2 is empty because CBS2 lacks a critical aspartate residue, which is required to make hydrogen bonds with the hydroxyl groups of the pentose sugar in the adenine nucleotides, while a non-exchangeable AMP molecule permanently occupies SITE-4.[35] Under physiological conditions, the concentration of ATP > ADP > AMP and most ATP molecules exist in complex with magnesium ion (Mg-ATP) while ADP and AMP do not.[36] The relative binding affinities (K_d) of the various adenine nucleotides for the exchangeable binding sites (SITE-1 and SITE-3) on the γ subunit are ATP : ADP : AMP : Mg-ATP = 0.9 : 1.3 : 1.6 : 32. Additionally, myristoylation of residues in the N-terminus of the β subunit in the presence of AMP-bound γ subunit modestly increases AMPK activity.[32] Changes in the cellular concentration of ATP, ADP or AMP will change occupancy of SITE-1 and SITE-3, which allows the γ subunit of AMPK to function as an energy sensor in cells.[36] Changes to the occupancy of the adenine nucleotide-binding site lead to short- and long-range conformational effects transmitted through the β subunit-binding site on the γ subunit.

A decrease in ATP levels due to metabolic stress (*i.e.* decreased glucose levels) or rapid and increased consumption of ATP (*e.g.* during muscle contraction), leads to an increase in the ADP : ATP ratio. As ADP levels rise, a reverse adenylate kinase reaction (2ADP ➔ ATP + AMP) will drive the synthesis of ATP and AMP. This will alter the cellular ATP : ADP : AMP ratio. An increase in cellular ADP and AMP levels will drive the displacement of ATP, which is found in high levels when the cells are not stressed, from SITE-1 and SITE-3 of the γ subunit of AMPK.[36]

Xiao *et al.* proposed that the α-hook region on the catalytic α subunit interacts with exchangeable SITE-3 on the γ-domain when an AMP/ADP molecule occupies it. This α-hook interaction enhances the recruitment of the kinase domain to the regulatory subunits. The interaction between the CBM domain of the β subunit and the activation loop of the kinase domain stabilizes the activation loop structure. This maintains the activated state of AMPK. AMP binding to SITE-1 and SITE-3 of the γ subunit allosterically increases AMPK activity 2–5 fold.[36] Recently, a full-length human α2β1γ1 AMPK crystal structure was reported, revealing that the phosphate group on Thr-172 is partially exposed to solvent and is not accessible to phosphatases.[37] For dephosphorylation to occur, the activation loop must undergo a conformational change that enables the phosphate group to be solvent exposed. The interactions of the regulatory fragments with the activation loop block the dephosphorylation of Thr-172.[36,37] Furthermore, AMP binding helps maintain AMPK in the activated state by decreasing the rate of Thr-172 dephosphorylation.[36] In addition to the enzyme active site and nucleotide binding sites, the AMPK trimeric complex offers an array of protein-protein interfaces (PPI's) that can be targeted to modulate AMPK function. We have used high throughput screening (HTS) and peptidomimetic approaches to develop chemical probes that target PPI's.[38–48] Similar strategies can be employed to develop inhibitors against the AMPK PPI's.

The modes of activation listed above are driven by conformational changes that either allow or block access to Thr-172. Another mode of AMPK activation that is independent of cellular adenine nucleotide levels is Ca^{2+}-mediated activation of the upstream kinase CaMKKβ.[25,26,49,50] Intracellular Ca^{2+} levels are tightly controlled in cells with the endoplasmic reticulum (ER) serving as the Ca^{2+} store of the cell. Phospholipases activated by cell surface receptors lead to inositol triphosphate-induced Ca^{2+} release from the ER.

AMPK-mediated signaling and its effects on metabolic pathways

Depletion of ATP activates AMPK as ADP and AMP begin to displace ATP from the γ subunit. Depending on the severity of ATP depletion, different events such as increased glucose uptake, increased glycolysis and reduced glycogen synthesis will be triggered to restore ATP levels. Glucose transporters (GLUT) are a family of membrane proteins that play an integral part in responding to and assisting in glucose uptake by cells. Genetic and pharmacological manipulation of AMPK in adipocytes, muscle and neurons suggests indirect regulation of GLUT expression and translocation by AMPK.[51–53] At this time the exact signal transduction pathway that leads to AMPK-driven GLUT-mediated glucose uptake is not fully understood. Given the diversity in the GLUTs and AMPK, it is highly likely that the signaling pathway that links these two proteins is tissue specific. Activation of glycolysis by AMPK is driven by isoform-specific phosphorylation and activation of phosphofructokinase (PFK).[17,54] Inhibition of glycogen synthesis by the activation of AMPK is driven by the phosphorylation and inactivation of glycogen synthases.[55] The severity of the metabolic stress will determine the level of activation as well as the number of processes activated to respond to the stress and restore normalcy.

Direct targets of AMPK

Since AMPK serves as the energy sensor in cells, it is not surprising that activation of AMPK leads to changes in a plethora of cellular functions. Acetyl-Co-Acarboxylase-1 (ACC1), Acetyl-Co-A-carboxylase-2 (ACC2), GS and 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase are well-characterized direct targets of AMPK. In this section we will limit the discussions to lesser-known and recently discovered direct targets. A kinase assay using truncated GST-fusion kinase domain of AMPK α-subunit and a GST-fusion ACC was established. A structure activity relationship (SAR) with 25 mutant GST-ACC revealed a > 20 amino acid interaction region between AMPK and ACC.[56] The study also predicted a consensus recognition sequence (xφxxφxxxφxxβφβxxx**s**xxxφ where φ = hydrophobic residue, β = basic residue and **s** = phosphorylation residue).[56,57]

AMPK is a negative regulator of phosphoenolpyruvate carboxykinase (PEPCK) gene expression (Figure 4). An early step in hepatic gluconeogenesis is PEPCK-catalyzed conversion of oxaloacetate to phosphoenolpyruvate.[61] AMPK modulators such as the small molecule AMPK activator AICAR, the hormone adiponectin and the antidiabetic drug metformin are known to reduce PEPCK gene expression.[62–64] In a systematic study Inoue *et al.* reported the identification of AICAR response element binding protein (AREBP) as a zinc finger transcription factor that acts as a repressor of PEPCK gene expression. An *in vitro* AMPK phosphorylation assay using a series of AREBP point mutants identified Ser-470 as the molecular target of AMPK and gel electrophoresis mobility shift assay demonstrated this phosphorylation prevents AREBP binding to DNA.[8] The model supports phosphorylation of Ser-470 by AMPK, which abolishes AREBP DNA-binding activity, as the molecular basis for the transcriptional repression of PEPCK gene expression by AREBP.

Protein synthesis involves three major steps, namely, initiation, elongation and termination. Among the three, >99% of the energy required for protein synthesis is used during elongation. In eukaryotic cells, peptide chain elongation requires two elongation factors, eEF1A and eEF2. A drop in cellular ATP levels leads to the phosphorylation of eEF2 by eEF2 kinase, which results in the inhibition of protein synthesis. AMPK serves as the link between cellular energy metabolism and regulation of protein synthesis, wherein AMPK directly phosphorylates eEF2 kinase at Ser-398. *In vitro* studies identified two additional sites, Ser-78 and Ser-366 that were phosphorylated by AMPK but to a lesser extent. However, only Ser-398 phosphorylation was observed in cells treated with AICAR, an AMPK activator.[4]

Nitric Oxide (NO) has been implicated as a modulator in different physiological processes such as mitochondrial respiration, glucose uptake, glycolysis and muscle contraction.[65] Inhibition of nitric oxide synthase (NOS) reduces glucose uptake. As AMPK is involved in the regulation of glucose uptake in skeletal muscle during exercise, a possible link between AMPK and NOS may exist. Chen *et al.* showed that endothelial nitric-oxide synthase (eNOS) was phosphorylated and activated by AMPK and mass spectrometry studies revealed Ser-1177 as the phosphorylation site. Similarly, neuronal nitric-oxide synthase-μ (nNOS-μ) containing Ser-1451 at a similar position was phosphorylated by AMPK.[14] A second site, Thr-495, on eNOS was also phosphorylated by AMPK *in vitro* in the absence of

Ca^{+2}/calmodulin resulting in inhibition of eNOS activity. However, during ischemia an increase in only Ser-1177 phosphorylation was observed.[10]

Using *in vitro* kinase assays, Hong *et al.* showed AMPK phosphorylates Ser-304 of the transcription factor hepatocyte nuclear factor 4alpha (HNF4α), increasing its degradation and reducing its ability to bind DNA. Introduction of a phosphoserine mimetic in HNF4α (S304D) resulted in decreased protein stability, dimerization, DNA binding and HNF4α-mediated transcription. A nonsense mutation in a single allele of HNF4α leads to maturity onset diabetes of the young (MODY). Patients with this inherited form of diabetes show defects in pancreatic function such as reduced insulin secretion.[12]

The insulin receptor substrate (IRS-1) contains multiple potential tyrosine phosphorylation sites with the consensus motif YMXM, which is recognized by the insulin receptor (IR) kinase. Proteins such as phosphatidylinositide 3-kinases (PI3K) bind to these sites via their Src homology 2 (SH2) domains, allowing IRS-1 to serve as a docking protein for SH2 domain-containing signal-transduction proteins.[66,67] Furthermore, IRS-1 contains multiple serine and threonine phosphorylation sites. Chopra *et al.* demonstrated phosphorylation of IRS-1 Ser-789 in cardiac myocytes following glucose starvation and also showed that this phosphorylation was mimicked by AICAR treatment. However, phosphorylation at this site negatively regulates the insulin pathway. On the other hand, phosphorylation of Tyr-612 and Tyr-632 residues of IRS-1 also occurred following glucose starvation and was mimicked by AICAR treatment. Both glucose starvation and AICAR treatment resulted in levels of phosphorylated Tyr-612 and Tyr-632 similar to those seen upon insulin stimulation. Treatment with compound C, an AMPK inhibitor, blocked phosphorylation of Tyr-632.[68] Furthermore, glucose starvation led to phosphorylation of insulin receptor (IR) Tyr-1162, which was inhibited by a dominant negative AMPK or compound C treatment. Overall, their studies suggest AMPK phosphorylates IR, which leads to allosteric activation of IR kinase and signal transduction through IRS-1 by direct binding of PI3K to Tyr-612/Tyr-632.[69] However, studies by Jakobsen *et al.*, suggest that phosphorylation of Ser-789 appears to potentiate the activity of PI3K associated with IRS-1 in C2C12 myotubes.[13]

$p27^{Kip1}$ regulates a number of cellular functions and chief among them is induction of cell-cycle arrest by disruption of the cyclin E - cyclin dependent kinase - 2 (CDK2) complex. Transfection with the phosphomimetic T198D mutant allele of p27 modestly inhibited colony formation compared to wild-type and both wild-type and T198D p27 induced G1 arrest compared to the non-phosphorylatable T198A p27. Under serum or glucose deprivation of cells, a robust increase in phosphorylated p27 was observed. This increase in Thr-198 phosphorylation correlated with an increase in AMPK and ACC phosphorylation, suggesting Thr-198 phosphorylation of p27 is regulated by AMPK. Studies by Liang *et al.* showed purified AMPKα1 phosphorylates recombinant p27 and that mutation of Thr-198 to alanine resulted in altered p27 stability, indicating that Thr-198 of p27 is a direct target of AMPK *in vitro*. Their studies suggested that under stress, phosphorylation of Thr-198 promotes p27 stability. Accumulation of p27 in quiescent cells dictates whether cells enter the autophagy-mediated cell survival pathway or undergo apoptosis.[15]

The transcriptional coactivator p300 regulates transcription by recruiting transcription machinery to promoters and linking DNA-bound transcription factors to the basal transcription machinery. Furthermore, p300 may regulate transcription by acetylation of transcription factors or by modification of chromatin structure via acetylation of histones.[70] Ser-89 on p300 is a target for phosphorylation by kinases including AMPK. Yang *et al.* demonstrated that Ser-89 on p300 is a direct substrate of AMPK. Using a mammalian two-hybrid system they showed that ligand-dependent peroxisome proliferator-activated receptor-γ (PPARγ)/p300 interaction-mediated transcriptional activity is reduced with a S89D p300 mutant. This is just one example that shows AMPK regulates gene expression in response to alterations in the energy and metabolic landscape.[16]

Marsin *et al.* demonstrated that under anaerobic conditions, such as ischemia, the AMP : ATP ratio increases, which lead to activation of AMPK and phosphorylation of heart phosphofructokinase-2 (PFK-2) at Ser-466. PFK-1, an enzyme that plays a key role in glycolysis, serves as a ubiquitous glycolytic signal and is directly stimulated by fructose 2,6-bisphosphate, which is synthesized by PFK-2. The net effect of PFK-2 phosphorylation by AMPK is the activation of glycolysis in the heart during ischemia. These studies show that phosphorylation of PFK-2 by AMPK is a secondary indirect mechanism of PFK-1 activation that leads to ATP production.[17]

Tuberous sclerosis complex (TSC) is an autosomal disorder caused by a mutation in either TSC1 or TSC2 tumor suppressor genes. TSC proteins negatively regulate translation through the mammalian target of rapamycin (mTOR) pathway. In cells, starvation activates TSC2, which leads to the phosphorylation of mTOR substrates ribosomal S6 kinase (S6K) and eukaryotic initiation factor 4E binding protein 1 (4EBP1). Two-dimensional phosphopeptide mapping and mutational studies showed that Thr-1227 and Ser-1345 on TSC2 are direct targets for phosphorylation and activation by the energy sensor AMPK.[18]

A chemical genetics screen was used to identify 28 new AMPK substrates. A subset of the substrates identified was validated using *in vitro* kinase assays. Follow up studies revealed that protein phosphatase 1 regulatory subunit 12C (PPP1R12C) and p21- activated protein kinase (PAK2) are direct targets of AMPK. Phosphorylation of Ser-20 on PAK2 and Ser-452 on PPP1R12C by AMPK promotes myosin regulatory light chain (MRLC)-mediated completion of mitosis (cytokinesis).[19]

A yeast two-hybrid screen of a human heart cDNA library using a truncated γ2 isoform of AMPK identified cTnI as a putative target for AMPK phosphorylation. *In vitro* studies identified Ser-150 as the site of phosphorylation by AMPK. AMPK activation of cTnI through Ser-150 phosphorylation was observed in whole hearts during ischemia.[59]

PIKfyve is a lipid kinase that phosphorylates phosphatidylinositides (PtdIns) to PtdIns5P and PtdIns3P to PtdIns(3,5)P_2. Several studies have implicated PIKfyve in insulin-stimulated GLUT4 translocation and glucose uptake.[71] Changes in the AMP : ADP : ATP ratios during muscle contraction activate AMPK and increases GLUT4 translocation and glucose uptake, suggesting a possible link between AMPK and PIKfyve in contraction-stimulated glucose uptake. *In vitro* studies using recombinant WT-PIKfyve, [γ-^{32}]ATP and AMPK suggest

direct phosphorylation of PIKfyve by AMPK. MS studies identified Ser-307 and Ser-48 as putative phosphorylation sites. Phosphorylation of PIKfyve Ser-307 did not affect its lipid kinase activity but did alter its subcellular localization, leading to its translocation to early endosomes and PtdIns(3,5)P_2 production, which may increase GLUT4 translocation.[60]

Role of AMPK in Cancer

Patients with metabolic dysfunctions, such as insulin resistance, type 2 diabetes and obesity, have a higher risk of developing cancer.[72] Under energetic stress, AMPK activates catabolic processes and inhibits anabolic processes, which results in the inhibition of cell proliferation. These observations led to speculation that AMPK is a tumor suppressor. In human cancer, however, AMPK mutations are rare, suggesting that loss of AMPK function is probably due to mutations in upstream kinases such as LKB1 or downstream targets like TSC2.[23,24,73,74] As such, activation of AMPK has been proposed as a treatment option for cancer. Epidemiological data suggest metformin, the clinically used glucose-lowering agent, may reduce cancer risk. Buzzai *et al.* showed that metformin-treated, colon tumor-bearing (HC116 $p53^{-/-}$ xenografts) mice showed reduced tumor sizes when compared to vehicle treated mice. This suggests metformin-induced cytotoxicity of colon cancer cells with $p53^{-/-}$ phenotype. *In vitro* studies suggested that only cells grown in the absence of glucose were sensitive to metformin treatment, suggesting that p53 inactivation impairs cell survival under nutrient deprivation, which would occur in tumors that are hypoxic and have low vascularization.[75] Germline mutations in the upstream kinase LKB1 prevent AMPK activation and cause Peutz-Jeghers syndrome (PJS), a risk factor for developing malignant tumors.[76] Inactivation of LKB1 leads to hyperactivation of mammalian target of rapamycin complex-1 (mTORC1), a cell growth regulator, which promotes cell growth and cell proliferation.[77] Dennis *et al.* reported that low nutrient conditions inhibit anabolic processes driven by mTOR signaling.[78] AMPK is known to directly phosphorylate TSC2, which negatively regulates translation through the mTOR (Figure 4).[18] These results suggest that LKB1 negatively regulates mTORC1 through phosphorylation of AMPK. Additionally, Tiainen *et al.* have demonstrated that LKB1 activation induces G1 arrest and up-regulation of $p21^{Kip1}$, a p53 target gene, in a p53-dependent manner.[79] Jones *et al.* later showed AMPK phosphorylates Ser-15 of p53 to induce cell-cycle arrest.[80] AMPK, among others, phosphorylates raptor leading to inhibition of mTORC1 complex activity.[18,81] TSC1 and TSC2 negatively regulate mTOR signaling and inactivation of either TSC1 or TSC2 is associated with elevated levels of mTOR activity and activation of the mTOR pathway is reported in TSC1 and TSC2-deficient tissues.[82–85] Despite these reports, since both LKB1 and AMPK phosphorylate a plethora of cellular substrates and mTOR serves as a signaling node for a wide range of cellular functions, the exact mechanism of LKB1-AMPK-mediated mTOR activation is not fully understood.

AMPK inactivity is also implicated in the switch to aerobic glycolysis by cancer cells. During glycolysis cells metabolize glucose to pyruvate in the cytoplasm to generate 2 ATP molecules/glucose. In the Krebs cycle, also known as the tricarboxylic acid (TCA) cycle, pyruvate generated from glycolysis is oxidized to acetyl-CoA and used to generate Nicotinamide adenine dinucleotide (NADH). This NADH produced in the Krebs cycle is then used during oxidative phosphorylation in the mitochondria to generate 36 ATP/glucose.

Under hypoxia lactate dehydrogenase (LDH) converts the pyruvate from glycolysis to lactic acid (anaerobic glycolysis). The ability of tumor slices to consume high levels of glucose and produce high levels of lactate *ex vivo* in the presence of adequate oxygen led Otto Warburg to postulate that a change in the metabolic landscape as the cause of cancer.[86] The Warburg effect, also known as aerobic glycolysis, has since been confirmed, however, activation of oncogenes and loss of tumor suppressors are implicated as the cause.[87] In the case of cancer, oncogene and tumor suppressor networks alter tumor cell metabolism to generate energy and biomass at higher rates to meet the demands of proliferation. AMPK serves as an ATP sensor in cells and AMPK signals to and from known tumor suppressors (TSC2 and LKB1). A recent study conducted by Faubert *et al.* explored the role of AMPK on the Warburg effect and tumorigenesis and concluded that inactivation of AMPK enhances aerobic glycolysis.[88] They knocked out the α1 subunit of AMPK (α2 is not expressed in B lymphocytes) in Eμ-Myc transgenic mice.[89] Both the homozygous Eμ-Myc/$\alpha1^{-/-}$ and heterozygous Eμ-Myc/$\alpha1^{+/-}$ mice displayed pre-B cell tumors with accelerated lymphomagenesis as opposed to mature B cell tumors found in Eμ-Myc/$\alpha1^{+/+}$ mice. These studies also found that HIF-1α is a key mediator of AMPK-dependent effects on cellular metabolism.[88] The significant increase in lactate production observed in these studies suggests that down regulation of AMPK signaling is sufficient to enhance the Warburg effect in cancer cells. This data suggests that activation of AMPK may be a viable therapeutic option for cancer.

On the other hand, the use of AMPK inhibitors may also be a viable treatment option for cancer. For example, in a study by Shaw *et al.* LKB1 knock out led to increased mTOR activity and increased cell proliferation, however, $Lkb1^{-/-}$ Mouse embryonic fibroblasts (MEFs), unlike other tumor suppressor gene knockout MEFs, underwent rapid apoptosis under energy stress. This phenotype was rescued by the mTOR inhibitor rapamycin.[76] Furthermore, in the study conducted by Faubert *et al.* knockdown of AMPKα1 using shRNAs in Eμ-Myc lymphoma cells resulted in sensitivity to metabolic stress induced by the glycolytic inhibitor 2-deoxyglucose (2-DG), suggesting that although AMPK favors the Warburg effect, it is also needed for metabolic checkpoints that allow cancer cells to adapt to stress.[88] These studies suggest that certain AMPK inhibitors may provide a therapeutic advantage when used in combination with agents that induce energetic stress. Additionally, activation of AMPK is reported to induce autophagy via inhibition of mTOR, a negative regulator of autophagy.[90,91] Hypoxia in prostate cancer activates AMPK, which functions to maintain cell survival. Using immunohistochemistry, Park *et al.* showed elevated levels of phosphorylated ACC, an AMPK substrate, in approximately 40% of human prostate cancer samples examined, thus implicating AMPK. Consistent with the above observation knockdown of the α1 and α2 subunits of AMPK in prostate cancer cell lines resulted in reduced proliferation. In the same study, prostate cancer cells treated with the AMPK inhibitor compound C (**1**, Figure 3) exhibited reduced cell growth and increased apoptosis.[92] This cell survival-promoting role of AMPK may give cancer cells an advantage during selection pressure created by the tumor microenvironment. Since AMPK activation is reversed when the energy balance is restored, AMPK has been suggested as a conditional oncogene.[93] AMPK activation may be essential for the survival of cancer cells early during tumorigenesis and studies support the notion that AMPK is required for anchorage-

independent growth and survival during glucose deprivation.[94] Though the seemingly paradoxical role of AMPK still remains partially unsolved, work by Jeon *et al.* demonstrates that AMPK promotes tumor cell survival by regulating NADPH homeostasis during energetic stress. Their study showed that AMPK functions to maintain levels of NADPH by inhibiting ACC1 and ACC2 and thereby inhibiting NADPH-consuming fatty acid synthesis and increasing fatty acid oxidation, during matrix detachment and when NADPH generation by the pentose phosphate pathway is decreased by glucose deprivation. They suggest that combinational treatment with AMPK activators and ACC activators may inhibit cancer cell survival, as this treatment strategy would drive AMPK inactivation of mTOR while blocking the regulation of NADPH homeostasis by AMPK.[95] Information regarding tissue-specific expression of AMPK subunits and the roles of AMPK in early and late tumorigenesis is needed to determine if inhibitors or activators of AMPK are appropriate for the treatment of cancer.

Role of AMPK in metabolic disease

Organs and tissues relevant to metabolic disorders include skeletal muscle, liver and adipose tissue. AMPK α2 knockout mice on a normal diet showed impaired glucose tolerance and reduced insulin-stimulated glucose metabolism. On a high-fat diet, these mice were glucose tolerant despite increased body weight and fat mass, highlighting the importance of AMPK in metabolic homeostasis.[96] Since loss of glucose tolerance, increased body weight and increased fat mass are the hallmarks of type 2 diabetes and obesity, the above study suggests AMPK activation is a viable therapeutic approach for the treatment of metabolic disorders.[97] Consistent with the above statement, pharmacological activation of AMPK by AICAR led to increased glucose uptake by muscles and inhibition of gluconeogenesis in the liver. The study concluded that AICAR treatment phenotypically mimics insulin-independent insulin action.[62] Treatment with N-(1-(4-cyanobenzyl)piperidin-4-yl)-6-(4-(4-methoxybenzoyl)piperidine-1-carbonyl)nicotin amide (R419, **2**, Figure 3), discussed later in the indirect activators section, leads to increased glucose uptake in myocytes and increased fatty acid oxidation in mouse primary hepatocytes.[98] Another study conducted by Baltgalvis *et al.* at Rigel pharmaceutical suggested that activation of AMPK by pharmacological small molecule activators offers a suitable treatment for intermittent claudication associated with peripheral artery disease (PAD). High-fat fed mice demonstrated characteristics of PAD and treatment of these animals with AMPK activator, R118 (structure not disclosed), alleviated some of these characteristics.[99] In a mouse model of diabetes, treatment with the indirect AMPK activator AdipoRon, also discussed later, leads to improved glucose tolerance and insulin resistance. The results from these studies suggest AMPK activation as a possible option for the treatment of diabetes.[100]

Role of AMPK in Lipogenesis

An emerging hallmark of cancer is increased rate of *de novo* fatty acid synthesis, which is a direct consequence of a tumor's shift towards glycolytic metabolism. Glucose taken up by a cell is converted into glucose-6-phosphate by hexokinases and is used to generate ATP and pyruvate. This pyruvate is converted to acetyl CoA and enters the mitochondria and the citric acid cycle. If oxygen is available, this acetyl CoA is converted to citrate, which will enter

oxidative phosphorylation. Under anaerobic conditions, however, this citrate is transported to the cytoplasm and reconverted by ATP citrate lyase to acetyl CoA, some of which is then converted into malonyl-CoA by ACC mediated carboxylation. Finally, fatty acid synthase condenses acetyl-CoA and malonyl-CoA to form saturated, long chain fatty acids, such as palmitate. These fatty acids can be further modified into phospholipids, triglycerides, and cholesterol esters, among others, which are primarily used to form the lipid bilayers of rapidly dividing tumor cells.[101] AMPK activators may target *de novo* fatty acid synthesis through AMPK's regulatory role in both the activation and expression of fatty acid synthesis proteins, such as ACC and fatty acid synthase. Winder *et al.* have shown that AMPK phosphorylates rat muscle ACC to increase its K_m for ATP and acetyl-CoA.[7] Swinnen *et al.* demonstrated that AICAR treatment of MDA-MB-231 breast cancer cells leads to AMPK activation, reduced lipogenesis, reduced DNA synthesis, and decreased protein synthesis.[102] Overall, AICAR treatment led to decreased cancer cell proliferation, migration, and invasion with increased cancer cell death. Additionally, AMPK-mediated ACC phosphorylation has been shown to regulate mitotic exit. Inhibition of fatty acid synthesis arrests cells between metaphase and telophase, suggesting AMPK activation in dividing cancer cells may inhibit cell division.[103] In fact, this phenomenon has been demonstrated in prostate cancer by Zadra *et al.*[104] Additionally, Vazquez-Martin *et al.* have shown that metformin treatment leads to mitotic catastrophe in cancer cells.[105] Overall, these studies suggest AMPK activation may indeed be beneficial for cancer treatment.

AMPK activation leads to fatty acid oxidation while blocking fatty acid and triglyceride synthesis, therefore novel activators of AMPK may prove useful for the treatment of metabolic disorders. Ruderman's group showed that AMPK activity is reduced in severely obese patients with insulin resistance and that AMPK activity is lower in visceral abdominal adipose tissue than in subcutaneous abdominal adipose tissue.[106] Furthermore, they showed decreased AMPK activity in a variety of animal models including the *fa/fa* (leptin-receptor-deficient, non-diabetic) and Zucker diabetic fatty (leptin-receptor-deficient, diabetes-prone) rats and *ob/ob* (leptin-deficient)[107] and Interleukin 6 (IL-6)-knockout mice.[108] Overall, these studies suggest that reduced AMPK activation may contribute to metabolic disease. Activation of AMPK with various small molecules for the treatment of metabolic disorders has led to modest success. For example, AICAR treatment of Zucker diabetic fatty rats prevents the development of diabetes and ectopic lipid accumulation.[107] Treatment of Dahl-S rats (a Sprague-Dawley strain with hypertriglyceridemia and high malonyl-CoA levels) with pioglitazone led to restoration of hepatic phosphorylated AMPK and phosphorylated ACC. Furthermore, treatment with pioglitazone led to decreased plasma triglyceride levels in Dahl-S rats.[109] The Erion group at Metabasis Therapeutics, Inc. reported the development of an AMPK activator (EC_{50} = 6.3 nM) that is > 900-fold more potent activator of AMPK than AMP (EC_{50} = 5.9 μM) and unlike 5-amino-4-imidazolecarboxamide ribotide (ZMP), it is inactive against glycogen phosphorylase (GPPase) and fructose-1,6-bisphosphatase (FBPase). Furthermore, ester and carbonate prodrugs of this AMPK activator inhibit *de novo* lipogenesis in rat hepatocytes with EC_{50} values < 1 μM, presumably through increased phosphorylation of ACC. In mice, these AMPK activators inhibited *de novo* lipogenesis by more than 30%.[110] However, the use of AMPK activators for the treatment of metabolic disease needs further investigation. Long-term (8 days) treatment of *ob/ob* mice with 0.25 –

0.5 mg/g AICAR improves glucose sensitivity of these mice, however, an increase in circulating triglycerides was also observed.[111]

Role of AMPK in Alzheimer's Disease

AD is a neurological protein misfolding disease, characterized by progressive dementia that leads to incapacitation and death. It includes synaptic loss and neuronal death, which over time are responsible for the loss of memory, personality changes and eventual death. There are two characteristic neuropathological lesions that define AD, namely, extracellular plaques and intracellular tangles.[112]

Extracellular plaques, also called amyloid plaques, are mainly composed of Aβ peptides, which consist of 39–43 amino acids that are proteolytic cleavage products of APP. The Aβ peptide segment is generated by the endoproteolysis of the transmembrane protein APP by beta (β) and gamma (γ) secretase enzymes. The conversion of Aβ from a soluble monomeric form to soluble aggregated forms appears to be the initial process of amyloid neurotoxicity.[113] Several studies demonstrated that AMPK plays an important role in the pathogenesis of Aβ generation.[114–116] Won *et al.* showed elevated levels of Aβ peptide in AMPKα2 knockout mice and activation of AMPK decreased Aβ production by regulating APP processing in lipid rafts. Their studies also showed that neurons from AMPKα2 knockout mice have elevated levels of cholesterol and sphingomyelin. Since cholesterol and sphingomyelin are associated with APP processing, which leads to Aβ production, AMPK may play a role in cholesterol and sphingomyelin regulation and APP processing in lipid rafts.[21] AICAR and other AMPK activators induced the opposite phenotype, wherein they show reduced accumulation of Aβ[117] whereas compound **1**, an AMPK inhibitor, treatment had the opposite effect.[118] On the other hand, treatment of N2a695 cells (N2a neuroblastoma cells) with the AMPK activator metformin increased Aβ generation, which was inhibited by compound **1** treatment. Also β-secretase (BACE1) promoter activity is upregulated by metformin treatment in these cells, suggesting a link between AMPK and BACE1.[119]

Intracellular tangles, also called neurofibrillary tangles (NFTs), are aggregates of Aβ and the microtubule associated protein tau.[120] Phosphorylation of tau protein in neuronal microtubules regulates its binding to tubulin. Phosphorylation of tau is required for neurite growth and axonal transport, however, hyperphosphorylation of tau leads to its self-aggregation into NFTs, the formation of which is another causative factor for AD.[121] Phosphorylation of tau is regulated by a series of kinases, such as stress-activated protein kinase,[122] CaMKKβ,[123] Glycogen synthase kinase-3-beta (GSK-3β),[124] cyclin-dependent kinase 5[124], and Src family tyrosine kinases.[125] Recent studies have identified recombinant AMPK as a tau kinase that is activated in response to amyloid Aβ peptide exposure.[126] AMPK phosphorylation of tau has been shown to prevent tau binding to microtubules, implicating tau in the formation of NFTs. Additionally, a link between AMPK, adenylate kinase-1 (AK1), and Aβ accumulation appears to exist in Alzheimer's disease.[127] Phosphorylation of AMPK Thr-172 appears to be reduced in patients with AD. Treatment of primary cortical neurons with Aβ42 leads to a reduction of AMPK Thr-172 phosphorylation and impairment of AMPK activity. Aβ42 also increases AK1 expression. Additionally, overexpression of WT AK1 leads to reduced AMPK Thr-172 phosphorylation while

overexpression of a mutant AK1 does not. Down regulation of the AMP/ATP ratio by AK1 may impact AMPK activity. As AMPK activation with AICAR leads to increased inhibitory phosphorylation of GSK3β and a decrease in tau phosphorylation, it is possible that AK1 drives tau phosphorylation through inhibition AMPK and as a consequence activation of GSK3β.[127] Following Ca^{+2}-dependent stimulation by Aβ(1–42) AMPK is phosphorylated by CaMKKβ.[126] Ca^{+2} homeostasis is critical for maintenance of synaptic plasticity, learning and memory, and disruption of Ca^{2+} homeostasis has been implicated in AD pathogenesis.[128]

On the other hand, studies have implicated tau acetylation, which results in inhibition of its degradation, in AD. Hyperphosphorylation of tau and the formation of NFTs occurred after tau acetylation in AD. NAD-dependent deacetylase sirtuin-1 (SIRT1), which is activated by AMPK, plays an important role in the reduction of tau acetylation and thus decreased tau hyperphosphorylation.[129] Additionally, studies have shown that the AMPK activator AICAR inhibits tau phosphorylation whereas, AMPK inhibition increases tau phosphorylation, further implicating AMPK activation in blocking tau phosphorylation.[130,131] Pharmacological targeting of AMPK may also be beneficial in other brain-related injuries and diseases. Several studies showed a direct correlation between AMPK activation and detrimental outcomes of experimental stroke probably due to ischemia-induced metabolic changes.[132–134] Hypothermia inhibited activation of AMPK in the brain, which resulted in neuroprotection following stroke in mice. Pharmacological inhibition of AMPK by **1** and AMPKα2 knockout prevented hypothermia-induced neuroprotection during experimental stroke, suggesting the protection provided by hypothermia is due to inhibition of AMPKα2.[135] In Huntington's disease, over activation of AMPKα1 potentiated striatal neurodegeneration.[136] Additional investigations into the brain specific role of AMPK are essential to determine if it is a viable target for AD and other neurodegenerative diseases.

Direct Activators

AICAR

AICAR (**3**, Scheme 1) was isolated as a crystalline compound from a sulfonamide-inhibited *Escherichia coli* culture in 1956.[137–139] Compound **3** is an inosine precursor and an adenosine analog that is transported across the cell membrane by adenosine transporters.[140] Compound **3** is phosphorylated in cells by adenosine kinase on the 5′-hydroxyl to generate 5-aminoimidazole-4-carboxamide ribonucleotide monophosphate (ZMP) (**4**, Scheme 1).[141] Initial reports argued that **3** treatment led to the accumulation of **4**, which inhibited adenylosuccinate AMP lyase (**5,** Scheme 1) and led to muscle dysfunction.[140,142,143] Sullivan *et al.* in 1994 showed that **4** stimulates human and rat AMPK and had a 20-fold better K_m than **5**. They concluded saying, "These novel activators of AMPK should prove useful in studying the role of the kinase in the regulation of cell metabolism."[144] Compound **4** binds to site 1 and/or site 3 of the regulatory γ subunit of AMPK and activates AMPK.[145] **4** and **5** bind to the same allosteric site on AMPK and have similar kinetic effects on AMPK activation isolated from rat liver.[146] Unlike other AMPK activators such as fructose, heat shock, and arsenite treatment, AMPK activation by **3** is independent of the AMP : ATP ratio making it an ideal probe to study the role of AMPK.[147] Structural studies with AMP analogs

(Figure 5) suggest that the free amino group at position 6 is important for stimulation of AMPK. Compared to parent **5** and **4**, 6-substituted AMP analogs such as 5-amino-4-imidazole-*N*-succinocarboxamide ribonucleotide (SAICAR) (**6**, Figure 5), adenyl-succinic acid (S-AMP) (**7**, Figure 5), 6-mercaptopurine riboside (6-SH-AMP) (**8**, Figure 5), 1, N^6-Ethenoadenosine- 5′-*O*- monophosphate (1,N^6-Etheno-AMP) (**9**, Figure 5) and N^6-Methyl-AMP (**10**, Figure 5) displayed no AMPK activation. Also, c-AMP (**11**, Figure 5) and c-ZMP (**12**, Figure 5) have no AMPK stimulating activity suggesting the importance of the monophosphate group. On the other hand, changes in the ribose moiety of 2′-deoxyadenosine-5′-monophosphate (dAMP) (**13**, Figure 5) and adenine-9-beta-*D*-arabinofuranoside 5′-monophosphate (Ara-AMP) (**14**, Figure 5) yielded partial activation. Changes in the adenine functionality in tubercidin 5′-monophosphate (Tu-MP) (**15**, Figure 5) also resulted in diminished enzyme activity.[146]

Compound **3** treatment results in the activation of AMPK through the phosphorylation of Thr-172 on its activation loop. Once activated, AMPK phosphorylates its downstream targets ACC, HMG-CoA reductase and fructose-1,6-bisphosphatase, among others (Table 1). These signaling cascades regulate numerous cellular functions that include fatty acid synthesis, cholesterol synthesis, gluconeogenesis and glucose uptake in skeletal muscle.[148–152] AICAR-stimulated glucose uptake was abolished in mouse muscle that expressed a kinase dead mutant (K46R) of AMPK establishing the link between **3** activation of AMPK and downstream cellular function (glucose uptake in muscle).[153] In the same study, glucose transport was only partially blocked in response to contraction suggesting the presence of parallel pathways that alter glucose flux into muscle. Rac1, the actin cytoskeleton-regulating GTPase, drives GLUT4 translocation in an insulin-dependent manner and this is an alternate pathway for contraction-stimulated glucose uptake.[154] Studies in mice and rat models consistently show that **3** treatment activates AMPK in different tissues and leads to the translocation of GLUT to the plasma membrane.[155] The observation that **3**-stimulated glucose transport is insulin independent led to preclinical studies that showed **3** treatment lowered blood glucose and improved glucose tolerance in the *ob/ob* mouse model.[149,156]

There are numerous studies that show **3** treatment leads to decreased cancer cell growth. Here we will limit the discussions to the studies that describe a mechanism of action. Among the many targets of AMPK, Ser-15 of p53 was identified to be present within a consensus recognition motif for AMPK.[80] Cells treated with **3** showed sustained p53-Ser-15 phosphorylation along with increased levels of CDK inhibitors $p21^{WAF/Cip1}$ and p27. In a panel of cancer cell lines, **3** induced cytostatic effects by arresting cells in the S-phase.[157,158] It is known that **3**-induced activation of AMPK leads to TSC2 phosphorylation-mediated mTOR inhibition.[81,159] In glioblastoma patients with mutant epidermal growth factor receptor (EGFR), transformation and signaling is driven by the mTOR/S6K pathway.[160,161] The use of rapamycin to block mTOR signaling failed in the clinics probably due to an protein kinase B (Akt) feedback loop.[162] An elegant preclinical study showed that despite only partial inhibition of mTOR signaling, compound **3** ability to block glucose uptake and lipogenesis made **3** a more effective therapeutic than rapamycin for EGFR mutant glioblastoma.[163]

Thienopyridone (A-769662 Abbott's compound)

The critical role of AMPK in the regulation of glucose and lipid metabolism led pharmaceutical companies to focus on the development of AMPK activators for the treatment of obesity and other metabolic diseases. Cool *et al.* conducted a HTS campaign to identify AMPK activators with a library of 700,000 compounds and a partially purified rat liver AMPK enzyme.[164] The agonist-induced phosphorylation of SAMS peptide (HMRSAMSGLHLVKRR) by AMPK was monitored. Non-nucleoside thienopyridone compound A-592107 (**16**, Scheme 2) was identified as a direct AMPK activator.[164] A lead optimization program starting with the original hit **16** (EC_{50} = 38 μM) led to the identification of a submicromolar compound A-769662 (**17**, Scheme 2) (EC_{50} = 0.8 μM).[164] Validation studies confirmed **17** is a reversible AMPK activator. The EC_{50} values for **17** were determined using partially purified AMPK extracts from rat heart, rat muscle and in human HEK cells.[164]

To determine if **17** acts as an AMP mimic *in vitro* studies were carried out using the enzymes glycogen phosphorylase (GPPase) and fructose-1,6-bisphosphatase (FBPase). **5** activated GPPase and inhibited FBPase.[165–167] However **17** had no effect on FBPase and GPPase suggesting that the mechanism of activation of AMPK by **17** is different from that of **5**. Consistent with the above observation, combination studies showed that **17** increased AMPK activity in the presence of saturating concentrations of **5**. Conversely, **5** stimulated AMPK in the presence of saturating concentrations of **17**. Although, like **5**, compound **17** activates AMPK by allosterically inhibiting Thr-172 dephosphorylation, the mode of activation by **17** is distinct from that of **5**. A systematic study with **17** showed that it activated only AMPK heterotrimers containing a β1 subunit.[168] Mutation R298G in the γ subunit, which abolished **5** activation, had no effect on compound **17** activation. On the other hand, mutation S108A in the regulatory β subunit completely abolished allosteric activation of **17** while sparing **5** activation.[169] A speculative model for activation of AMPK by **17** suggests that it binds to the glycogen-binding domain in the β subunit and stabilizes the conformation of AMPK that is resistant to Thr-172 dephosphorylation.[169] The model does not show direct interaction with the catalytic α subunit or the autoinhibitory domain. Together these suggest that **17** is a cell permeable AMPK activator and the mechanism of AMPK activation by **17** is different from that of **5**. Recently, Xiao *et al.* solved the structure of full-length human α2β1γ1 AMPK bound to small molecules **17** and 991 (**18**).[37] As anticipated, activator **17** sits at the interface between the N-terminal kinase domain and the CBM (Figure 6).

In vitro treatment of primary rat hepatocytes with **17** increased ACC phosphorylation and inhibited fatty acid synthesis.[164] Additionally, Sprague-Dawley (SD) rats treated with **17** resulted in increased fatty acid utilization and partially reduced malonyl CoA levels. Chronic treatment of *ob/ob* mice with **17** led to decreased plasma glucose and triglycerides, decreased expression of gluconeogenic enzymes and decreased weight gain compared to vehicle control.[164]

Following the work of Abbott laboratories, Merck GmbH identified thienopyridone compounds as AMPK activators for the treatment of diabetes, metabolic syndrome related

disorders and obesity.[170] Initial screening with two different fluorescent based technologies, AlphaScreen and Delfia, using the synthetic peptide substrate (AMARAASAAALARRR) yielded compound **19** (Figure 7) as an AMPK activator that increased basal AMPK activity by > 300%. Substitution of the methyl group at 5-position with a methoxy biphenyl **20** (Figure 7) showed a marginal increase in AMPK basal activity (111% at 30 μM) whereas analog **21** (Figure 7) with a hydroxy naphthalene substitution displayed better AMPK activation (625% at 30 μM).[171,172] Additional scaffolds reported by Merck GmbH include compound **22** (Figure 7), a 4-hydroxyimidazole-5-carboxamide (like **3**) substituted diphenyl urea, which showed > 310% increase in basal AMPK (purified from rat liver) activation at 200 μM concentration.[173] With the availability of the crystal structures and the limited SAR presented above, the thienopyridone core could be revisited as a viable starting point for a structure guided optimization of AMPK activators.

Mercury Therapeutics reported the synthesis and screening of hydroxybiphenyl compounds as AMPK modulators for the treatment of cancer, diabetes, and neurological diseases.[174] AMPK activity was measured by phosphorylation of an N-terminal fragment of human ACC1. This resulted in the identification of five biphenyl compounds (**23** – **27**) (Figure 7) with ED_{50} values < 10 μM.

Pyrrolopyridones

Using the Abbott compound **17** as a template, Mirguet *et al.* at GlaxoSmithKline (GSK) identified pyrrolopyridone analogs as a novel class of AMPK activators.[175] The major goal of the GSK group was to improve oral absorption and the selectivity profile. Compound **17** is an AMPK activator that is selective for β1-containing heterotrimeric complexes with poor oral bioavailability. The thiophene ring was targeted for replacement to improve oral bioavailability. A bioisosteric replacement of the thiophene ring in **17** with a pyrrole yielded compound pyrrolo[3,2-*b*]pyridine-5(*4H*)one (**28**, Scheme 3) with better oral bioavailability. However, **28** also showed higher blood clearance. A series of analogs with varying functional groups at the R_1, and R_2 positions were synthesized to reduce blood clearance and increase potency. A 3-methoxy-2-phenol substituent at the R_2 position resulted in a potent compound with reduced blood clearance but poor oral bioavailability. Substitution of hydrogen with chlorine (**29**, Scheme 3) in pyrrole ring did not improve the permeability or blood clearance but improved oral bioavailability. Substitution of the phenol with thiophene at R_2 position yielded compound **30** (Scheme 3) with good oral exposure and bioavailability.[175] The presence of the cyano group at the R_1 position on the pyridone ring was associated with poor permeability due to the acidic nature of the 3-cyanopyridone ring. Replacing the cyano group at R_1 with phenyl substitution improved permeability with good oral bioavailability (**31,** Scheme 3). Several analogs in this series showed improved blood exposure when co-dosed with a P-glycoprotein (P-gp) inhibitor suggesting that they may susceptible to Pgp-mediated efflux.[175]

Abbott compound **17** is selective for β1-containing AMPK heterotrimers.[168] On the other hand, compound **29** activates seven (α1β1γ1, α1β1γ2, α1β1γ3, α2β1γ1, α1β2γ1, α2β2γ1, α2β2γ3) of the twelve possible AMPK heterotrimers with pEC_{200s} in the lower μm range

(Table 2). Oral treatment of *ob/ob* mice with compound **31** (30 mg/kg for 5 days, bid) showed a 17% drop in blood glucose levels.[175]

Using phosphorylation of SAMS peptide as a readout for AMPK activation, a research group at Metabasis Therapeutics screened a focused library of 1,200 AMP mimetics and discovered compound **32** (Figure 8) as a potent and selective AMPK activator.[110] Compound **32** activated human AMPK with an EC_{50} of 6.3 nM, which is > 900 fold better than the endogenous activator **5** (EC_{50} = 5.9 μM). To overcome the poor cellular permeability, due to the charged nature of compound **32**, the Erion group designed different esterase-sensitive phosphonate prodrugs (**33** – **38**, Figure 8).[110] ACC is a direct substrate of AMPK and activation of AMPK leads to phosphorylation-mediated inactivation of ACC. Inactivation of ACC resulted in reduction of malonyl Co-A, and inhibition of *de novo* lipogenesis (DNL). Therefore compounds **33** – **38** were evaluated *in vitro* and *in vivo* for inhibition of DNL, and the results from this study are summarized in Table 3. Compounds were dosed (30 mg/kg) to C57BL/6 mice one hour prior to intraperitoneal administration of ^{14}C-acetate in saline. After one hour, newly synthesized lipids and sterols in liver and plasma were quantified and compared to vehicle control.[110] The limited SAR led to the identification of prodrugs with nM EC_{50} values and > 70% inhibition of DNL with a high correlation (R_2 > 0.95).

Benzimidazoles

A series of patent applications were filed claiming benzimidazoles **39** – **48** (Figure 9) are therapeutically relevant AMPK activators for the treatment, prevention, and suppression of diseases susceptible to AMPK activation.[176–180] AMPK activity was measured using α1β1γ1 recombinant human AMPK as half-maximal effective concentrations (EC_{50} relative to max activation by AMP) and activation effect relative to maximal activation by AMP (Act_{max}) respectively. A crystal structure of full-length α2β1γ1 AMPK complex with a small molecule activator **18,** which has a benzoxazole core is reported.[37] Compound **18** binds at the interface of α-kinase domain and the CBM of the β-subunit and preferentially activates AMPK complexes containing β1-subunits and binds with 10-fold higher affinity than **17**.[37]

PT1

Using an inactive form of truncated AMPK α subunit, Pang *et al.* screened a library of 3,600 compounds for their ability to activate this inactive form of the catalytic α subunit ($\alpha1^{(1–394)}$ containing the autoinhibitory domain). This led to the discovery of a small molecule activator (PT1) (**49**, Scheme 4) with an EC_{50} ~ 8 μM.[181] Follow up studies showed that **49** did not increase AMPK activity of the truncation mutant α1(1–312) lacking the autoinhibitory domain. A plausible mechanism for AMPK activation by **49** is conformational change-induced dissociation of the AID domain from the catalytic domain of the α subunit.[181] Subsequent studies confirmed that **49** interacts with the AMPK heterotrimeric complex α1β1γ1 in a dose-dependent manner (EC_{50} ~ 0.3 μM). A similar activation effect was observed when inactive AMPK α2 was treated with **49** with an EC_{50} ~ 12 μM. Docking studies suggest electrostatic interactions of **49** with Glu-96 and Lys-156 near the autoinhibitory domain in α1 subunit, relieve the autoinhibitory conformation and activate AMPK. When treated with **49**, other AMPK-related protein kinases such as human MAP/microtubule affinity-regulating kinase 2 (MARK2), BR serine/threonine kinase 1

(BRSK1), NUAK family SNF1-like kinase 2 (NUAK2) and maternal embryonic leucine zipper kinase (MELK) showed no change in activity, suggesting **49** is selective for the α subunit of AMPK. Treatment of L6 myotubes with **49** activated AMPK in a dose-dependent and time-dependent manner without changing the AMP : ATP ratio suggesting **49** is a direct AMPK activator. However, due to poor bioavailability and/or insufficient potency, **49** was inactive *in vivo*.[182]

In an effort to improve potency, a series of **49** analogs were synthesized and screened for AMPK activation.[182] Replacement of the central 2-imino-4-thiazolidone with a 3-alkylideneoxindole ring system yielded **50** (Scheme 4). Compared to **49**, compound **50** displayed a 3-fold increase in AMPK activity and a 5-fold increase in potency (EC^{50} = 2.1 μM). Further structural modifications resulted in the potent analog **51** (Scheme 4), which showed > 4-fold improvement in EC_{50} value over **49** in an AMPK activation assay and demonstrated beneficial metabolic effects in a diet-induced obesity model. A 4-week oral administration of **51** in *db/db* mice showed reduction of plasma triglycerides and improved glucose tolerance when compared with metformin.[183] A series of alkene-oxindole compounds (**52** – **55,** Figure 10) and 1,2,3,4-tetrahydroquinoline compounds (**56** – **58,** Figure 10) as AMPK activators were also reported by Hoffmann-La Roche AG for the treatment of diet-induced obesity and type 2 diabetes.[184–187]

Salicylate

Salicylate, an active component of willow bark, is a hormone produced by plants to fight infection.[188] Its synthetic derivatives such as aspirin and salsalate have been used towards the treatment of headache, lower back pain, osteoarthritis, and type 2-diabetes. Activation of AMPK was observed when HEK293 cells were treated with 1 mM of salicylate.[189] To test whether effects of salicylate are due to changes in ATP, ADP and AMP levels, isogenic cells expressing wild-type (WT) AMPK or mutated AMPK (R531G γ2 subunit, a mutation which renders AMPK insensitive to AMP or ADP) were treated with salicylate, which activated AMPK to the same extent without changing the ADP : ATP ratio suggesting an AMP-independent mechanism.[34] Concentration dependent studies suggest that at lower concentrations, AMPK phosphorylation and activation is independent of AMP and ADP levels, however at higher concentrations partial AMP and ADP dependent effects were observed.[189] Under physiological concentrations of ATP, salicylate caused 1.6-fold activation of AMPK with half-maximal effect at 1.0 ± 0.2 mM. Competition studies suggest that salicylate binds to the same site as activator **17** and similar protection against dephosphorylation and inactivation by phosphatases was observed. Increased fatty acid oxidation that was associated with phosphorylation and activation of AMPK was observed in isolated WT hepatocytes of salicylate-treated WT mice. Furthermore, phosphorylation of liver AMPK, soleus muscle AMPK and adipose tissue AMPK was observed in salicylate-treated WT mice but not in β1-KO mice.[189] Aspirin, a synthetic derivative of salicylate reduced mTOR signaling in colorectal cancer cells by inhibiting mTOR effectors S6K1 and 4E-BP1 and increased AMPK and ACC phosphorylation.[190]

Sanguinarine

In search of therapeutically relevant AMPK activators, Choi *et al.* screened a diverse library of 1,200 compounds using an *in vitro* fluorescence resonance energy transfer (FRET) assay.[191] Sanguinarine (**59,** Figure 11), a benzophenanthridine alkaloid, was identified as a validated hit.[191] Follow up studies with recombinant AMPK heterotrimers showed that **59** activated only AMPK heterotrimers that contained both the α1 and γ1 subunits and was ~5–10 fold less potent than AMP.[191]

Indirect Activators

AMPK serves as a signaling hub that can be activated by different modes: (i) allosteric activation by AMP and ADP, (ii) activation by upstream kinases, (iii) deactivation by phosphatases, (iv) conformational change to deactivate the autoinhibitory domain and (v) compounds that increases the AMP : ATP ratios within the cells are termed indirect AMPK activators. Well-characterized upstream kinases of AMPK include LKB1, CaMKKβ and Tak1. However additional yet-to-be-defined kinases may activate AMPK. Furthermore, the phosphatases that deactivate AMPK and all the AMPK complexes found in cells are not yet fully defined. Consequently, the mechanisms of action of many of the indirect activators listed below are not known. Nevertheless, treatment with these compounds ultimately leads to indirect activation of AMPK and alteration of the energy or metabolic landscape.

Metformin (**60**, Figure 12) a biguanide derivative of guanidine, is an antihyperglycemic agent. It is widely used for the treatment of type 2 diabetes and acts by suppressing hepatic glucose production.[192] Some studies suggest that **60** reduces the rate of gluconeogenesis,[193,194] while others suggest its mechanism of action is increased glucose uptake by skeletal muscle.[195,196] Studies by Zhou *et al.* established a connection between AMPK and **60**.[68] In rat hepatocytes, **60** treatment activated AMPK, which phosphorylated and reduced ACC activity. Inactivation of ACC reduced expression of sterol regulatory element binding protein-1 (SREBP-1), a lipogenic transcription factor, which led to the suppression of lipogenesis. Elevated levels of SREBP-1 are associated with insulin resistance in type 2 diabetes.[197] AMPK activation by **60** is dependent on the presence of LKB1. Shaw *et al.* showed **60** treatment lowered blood glucose by 40% in $LKB1^{+/+}$ *ob/ob* mice and no such effect was observed in the LKB1 null mice.[198] **60** is also a substrate of organic cation transporters (OCT). OCT1 plays a critical role in hepatic uptake of metformin and genetic ($OCT1^{-/-}$) and chemical (ethynylestradiol-induced cholestasis) disruption of OCT1 (expressed abundantly in the liver) function leads to reduced **60** distribution and consequently lowers its efficacy in reducing blood glucose levels.[199,200] OCT1 is highly polymorphic, in clinical studies individuals carrying OCT1 polymorphisms that resulted in reduced function had a poor response to **60** effects in glucose tolerant tests.[200]

Resveratrol (**61**, Figure 12) is a polyhydroxy-substituted stilbene found in several natural sources including the skin of red grapes. Studies by Vingtdeux *et al.* suggest **61** increases intracellular Ca^{+2} levels, which activates the kinase CaMKKβ that in turn phosphorylates and activates AMPK, although additional studies are needed to validate this mechanism of action.[201] Activation of AMPK by **61** resulted in mTOR inhibition, Aβ clearance in mice

and potentiation of autophagy. **61**-mediated activation of AMPK has thus been suggested as a therapeutic strategy to combat AD. A library of 158 compounds, structurally similar to **61**, were screened at 10 μM in APP-transfected HEK293 cells for their ability to reduce amyloid Aβ levels.[202] Like **61**, its analogs are not direct activators of AMPK but instead perturb upstream effectors, which lead to the activation of AMPK. Unlike **60**, compound **61** analogs were able to induce phosphorylation of AMPK and ACC in LKB1-deficient HeLa cells, suggesting that activation of AMPK by **61** is LKB1 independent. On the other hand, AMPK activation and ACC phosphorylation by the **61** analogs was dampened by co-treatment with CaMKKβ inhibitor. **61** has been reported to activate sirtuins and increase cell survival by stimulating SIRT1-dependent deacetylation of p53.[203] **61** increased human SIRT1 activity, but not the activity of other human Sir2 homologs (SIRT2), in fluorophore-labeled acetylated p53 derived peptide substrates.[204,205] Subsequent studies with full-length substrates in cell-free assays suggest indirect activation of SIRT1 by **61**.[206,207] Desquiret-Dumas *et al*. showed that **61** activates SIRT3 through an increase in NADH oxidation by complex 1.[208] Although the exact mechanism of action of **61** is not clear, **61** analogs have been shown to inhibit the mTOR pathway, induce autophagy and promote Aβ degradation by the lysosomal system in cells to lower Aβ accumulation/deposition in mice.[201]

Quercetin (**62**, Figure 12) is a flavanoid that is commonly found in a variety of fruits and vegetables. In isolated rat adipocytes, **62** inhibited methylglucose uptake with a Ki of 16 μM.[209] 3T3-L1 preadipocytes treated with **62** showed induction of AMPK phosphorylation in a dose-dependent manner. ACC is a known substrate of AMPK and phosphorylation of ACC inhibits adipogenesis. **62**-treated 3T3-L1 adipocytes showed decreased extracellular signal-regulated kinases (ERK) and c-Jun N-terminal kinases (JNK) phosphorylation and increased apoptosis.[210] Recent reports demonstrate that treatment with **62** decreases the expression levels of transcription regulators such as CCAAT/enhancer binding protein (C/EBP), alpha (C/EPBα) and PPARγ thereby suppressing the differentiation of preadipocytes to adipocytes.[211] However, the exact molecular mechanism underlying **62**-induced effects on adipocytes remain unclear.

The rhizomes of *Polygonatum odaratum* have been used as a traditional medicine and are commercially sold as food supplements. Guo *et al.* isolated homoisoflavanoids and dihydrochalcone from the rhizomes of *Polygonatum odaratum* (Mill.) Druce.[212] Compounds **63** – **66** (Figure 12) showed a significant increase in the phosphorylation of AMPK as well as the downstream substrate ACC. Pharmacological studies have demonstrated hypoglycemic effects with *P. odoratum* in diabetic animal models.[213,214]

Berberine (**67**, Figure 12) is a botanical alkaloid found in the roots and bark of several plants such as *Berberis vulgaris, Berberis asitata* and *Coptis chinensis,* among others. **67** is reported to have antihyperglycemic properties, antifungal, antiviral and antimicrobial activites.[215–221] In adipocytes, **67** treatment alters the AMP : ATP ratio, which leads to LKB1 and CaMKKβ independent activation of AMPK.[28, 222] Treatment with **67** reduced oxygen consumption in isolated muscle mitochondria containing complex I.[223] Other studies also suggest that **67** targets respiratory complex I.[219,224–227] 3T3-L1 adipocytes and L6 myotubes subjected to **67** treatment showed significant reduction in oxygen consumption suggesting a switch to

anaerobic respiration in cells.[223] Like other AMPK activators, **67** treatment resulted in increased glucose uptake in an insulin pathway independent manner. In a diet-induced obesity model, five weeks of **67** treatment significantly reduced fasting blood glucose and fasting insulin levels and improved insulin sensitivity.[228] A **67** derivative dihydroberberine (dhBBR) (**68,** Figure 12) showed improved oral bioavailability while phencopying berberine-induced effects.[223]

Thiazolidinediones (TZDs) such as rosiglitazone (**69**, Figure 12), troglitazone (**70**, Figure 12), and pioglitazone (**71**, Figure 12) are insulin-sensitizing agents commonly used for the treatment of type 2 diabetes. TZD compounds are high affinity ligands of the transcription factor PPAR-γ, which belongs to the nuclear hormone receptor superfamily.[229,230] Through PPAR-γ, TZDs modulate the transcription of critical genes involved in preadipocytes differentiation and fatty acid synthesis and storage.[231] TZD and their analogs also possess anticancer effects that are independent of PPAR-γ.[232–234] Compound **69** is a member of the thiazolidinedione class of oral antidiabetic drugs and improves insulin sensitivity and glucose homeostasis in type 2 diabetes patients.[235,236] The exact mechanism by which **69** improves insulin sensitivity and alters lipid and glucose metabolism remains poorly understood. Studies from the Carling group suggest that **69** activates AMPK in muscle by alterating the AMP : ATP ratio.[237] Treatment of H-2K^b muscle cells with **69** leads to activation of AMPK as inferred by phosphorylation of its substrate, ACC.

Guh *et al.* screened a focused library of in-house thiazolidinedione-based compounds and identified **72** (Figure 12) as a novel AMPK activator. Compound **72** activates AMPK in an LKB1 independent manner and inhibits lipopolysaccharide (LPS)-induced interleukin-6 (IL-6) production in human THP-1 cells.[238] Cytokine IL-6 plays an important role in the production of signal transducer and activator of transcription 3 (Stat3), which is constitutively active in 50% of primary breast tumors and is associated with poor prognosis.[239] *In vitro* and *in vivo* studies demonstrated that **72** is a potent antitumor agent that downregulates mTOR signaling through AMPK activation. The exact mechanism of AMPK activation by **72** is unclear, however, electrostatic potential map suggests that **72** might mediate AMPK activation *via* allosteric binding.[238] Direct AMPK activators that do not rely on LKB1 function have the potential to alleviate pathological conditions induced by LKB1 dysfunction.[240]

Epigallocatechin-3-gallate (EGCG) (**73**, Figure 12) is a natural compound found in green tea and has been suggested as a food supplement for the treatment of diet-induced obesity and type 2 diabetes.[241–243] Cellular studies show **73** treatment leads to inhibition of hepatic gluconeogenesis[244] and apoptosis in cancer cells.[245] However, limitations such as stability under physiological conditions, poor bioavailability and lower potency hinder its usage.[246,247] Compound **73** is unstable under physiological pH and tends to undergo methylation.[246] In order to improve its stability, the reactive hydroxyl groups of **73** were acetylated and prodrug (**74**, Figure 12) of EGCG with improved bioavailability was developed.[248] A focused library of epigallocatechin analogs was synthesized by replacing the reactive hydroxyl groups with H, OH, OAc, NH_2, alkyl, and halogens, among others. Evaluation of these analogs led to the identification of two new analogs (**75** and **76**, Figure 12) with improved AMPK activity.[249]

Curcumin (**77**, Figure 13) is a natural polyphenolic compound found in the rhizomes of turmeric and has a broad range of biological activities including anticancer activity. Studies by Pan *et al.* showed that **77** treatment of CaOV3 ovarian cancer cells increased phosphorylation of LKB1, ACC, p38 and p53.[250] Studies suggest that **77** activates LKB1-AMPK pathway, which results in cytotoxic effects in ovarian cancer cells. The α,β unsaturated ketones in curcumin makes it susceptible to nucleophiles in the cellular matrix.[251] The curcumin derivative, dimethoxycurcumin (DMC) (**78**, Figure 13) is a more stable and a potent activator of AMPK.[252]

α-lipoic acid (ALA) (**79,** Figure 13) is a naturally occurring plant-derived antioxidant that increases glucose uptake in skeletal muscle, increases insulin sensitivity in type 2 diabetes patients and reduces blood glucose levels. Recent studies have suggested that the increase in insulin sensitivity upon **79** treatment is due to activation of AMPK.[253] In **79**-treated C2C12 myotubes increased intracellular Ca^{2+} was observed. This led to the activation of CaMKKβ, an AMPK upstream kinase. Co-treatment with STO-609, a calcium chelator, blocked **79** induced AMPK activation.[254] Preclinical studies in animals showed that treatment with **79** reduced lipid accumulation.[255,256] Studies by Park *et al.* demonstrated that hepatic steatosis induced by high fat diet or liver X receptors (LXRs) agonist was blocked by **79** treatment. Compound **79** treatment also decreased the expression of SREBP-1c expression in these animals. Although 7**9** prevent fatty liver diseases its mechanism of action is yet to be fully defined.[257]

Arctigenin (**80**, Figure 13) is a phenylpropanoid dibenzylbutyrolactone isolated from the seeds of *Arctium lappa L.* Screening of an in-house natural product library by Tang *et al.* identified **80**, which activates AMPK both *in vitro* and *in vivo*.[258] A cell-based assay revealed that **80** promoted AMPK phosphorylation selectively at Thr-172 through the upstream kinases LKB1 and CaMKKβ. In an effort to improve potency and build a SAR, **80** analogs were synthesized and screened for AMPK phosphorylation in L6 myoblasts incubated with the analogs (40 μM) for 30 min. This led to the identification of additional analogs (**81** – **83,** Figure 12) with improved activity.[259]

5,7-Dihydroxy-2-(4-hydroxyphenyl)-6-methoxy-4*H*-1-benzopyran-4-one (Hispid ulin) (**84**, Figure 13) is a naturally occurring flavone found in *Saussurea involucrate,* a traditional Chinese herb with a range of biological activities.[260] Treatment of SKOV3 ovarian cancer cells with **84** resulted in activation of AMPK, inhibition of mTOR and significantly reduced Mcl-1 levels. Interestingly, **84** enhanced tumor necrosis factor-related apoptotic-inducing ligand (TRAIL)-induced apoptosis in SKOV3 cells providing a rationale for the combined use of AMPK activators and death receptor (DR) ligands for cancer therapy.[261]

Using HTS**,** Sviripa *et al.* identified *N,N′*-diarylurea (**85**, Figure 13), as an AMPK activator.[262] Several *N,N′*-diarylureas such as regorafenib, and sorafenib are used for the treatment of metastatic colorectal cancer, kidney cancer, and advanced liver cancer.[263,264] Treatment of LS147T colon cancer cells with **85** increased the phosphorylation of AMPK without changing the overall AMPK levels.

In search of natural AMPK activators, Kim *et al.* screened a natural product library and found panduratin (PAN) A (**86**, Figure 13), a compound isolated from the rhizomes of *Boesenbergia pandurata*.[265] Compound **86** treatment increased phosphorylation of AMPK and ACC in 3T3-L1 adipocytes, HepG2 liver carcinoma cells and L6 skeletal muscle cells. This effect was abolished by treatment with a AMPK inhibitor **1**.[68] Furthermore, activation of AMPK by **86** was completely abolished in LKB1-deficient cells suggesting LKB1-dependent AMPK activation. In addition, **86** altered AMPK subcellular localization and activated PPARα/γ. Oral administration of **86** to C57BL/6J mice on a high fat diet reduced triglycerides, total cholesterol and low-density lipoprotein cholesterol.[265]

Seeing the beneficial effects of AMPK activation towards the treatment of type-2 diabetes and obesity, Oh *et al.* screened a library of 2500 compounds and identified a small molecule AMPK activator, ampkinone (**87**, Figure 13).[266] *In vitro* studies suggested compound **87** activated AMPK and subsequent phosphorylation of ACC substrate. Immunocomplex kinase assays with **87** (10μM) led to a 2.7 fold increase in AMPK activity towards the SAMS peptide whereas 1mM **3** treatment induced 3.2 fold AMPK activation. Follow up studies suggests **87** as an indirect AMPK activator. Consistent with *in vitro* studies, **87** increased pAMPK and pACC levels in liver cells of diet-induced obese (DIO) mice. Lower glucose levels were observed in **87**-treated mice, which indicate that **87** is an AMPK activator with anti-diabetic effect.[266]

Tan *et al.* isolated two different classes of curcubitane triterpenoids from *Momordica charantia* (bitter melon).[267] Compounds **88** and **90** (Figure 13) and their aglycones **89** and **91** (Figure 13) activated AMPK in L6 muscle cells and 3T3L1 adipocytes in a dose-dependent manner and stimulated translocation of GLUT4 to the plasma membrane. Concentrations required for **88** – **91** are 10,000 times lower than compound **3** suggesting curcubitane triterpenoids are highly efficacious stimulants of GLUT4 translocation. No activation of AMPK in HeLa cells lacking LKB1 was observed suggesting that the mechanism of action of these curcubitane might be similar to metformin.[267] Recently, Chen *et al.* isolated new curcubitane triterpenoids (**92** – **94**, Figure 13) and reported them as potent AMPK activators in HepG2 cells.[268]

Adipocytes secrete adiponectin, which bind to AdipoR1 and AdipoR2 to activate AMPK and exert antidiabetic effects. In a screen of the chemical library, Chen *et al.* identified a small molecule activator of AdipoR, termed AdipoRon (**95**, Figure 13) that binds AdipoR1 and AdipoR2.[100] Surface plasmon resonance studies showed **95** binds both AdipoR1 and AdipoR2 with a K_d 1.8 and 3.1_μM, respectively. Increased AMPK Thr-172 phosphorylation was seen in C2C12 myocytes treated with **95** and was almost completely lost by treatment with AdipoR1 siRNA. In *db/db* mice on normal chow diet, treatment with **95** improved glucose intolerance and insulin resistance, suggesting AdipoR stimulation may be a viable therapeutic option for diabetes treatment.[100]

Small molecule R419 (**2**, Figure 1) was identified by Jenkins *et al.* as an AMPK activator that, like metformin, inhibits complex I. The small molecule activates liver, muscle, and adipose AMPK. Using ACC Ser-79 phosphorylation as a readout, the EC_{50} for the compound in HepG2 and C2C12 myotubes was estimated at ~ 0.03 and 0.23 μM,

respectively. Compound **2** treatment resulted in increased glucose uptake in myocytes, possibly through increased GLUT4 expression, and increased fatty acid oxidation in mouse primary hepatocytes, highlighting the potential therapeutic value of AMPK activation and regulation of mitochondrial function for the treatment of diabetes.[98]

Meltzer-Mats *et al.* used **49** as a starting compound and truncated it sequentially to identify benzothiazole (**96**, Scheme 5) as the minimally active moiety (100 μM treatment leads to 1.3 fold increase in glucose uptake) that is required to induce AMPK phosphorylation and glucose uptake in L6 myotubes.[269] A second fragment capable of activating AMPK was also identified (**97**, 1.8 fold at 100 μM, Scheme 5). A series of benzothiazole derivatives that combined the two fragment cores were synthesized and screened for glucose uptake and AMPK phosphorylation in L6 myotubes. Compound **98** (Scheme 5) was identified as an efficacious and potent AMPK activator that induced a 2.5 fold increase in glucose uptake at 100 μM. *In vivo* efficacy of compound **98** was evaluated in the KKAy mice model, which had previously been reported to develop diabetic traits similar to human patients.[270] In this model, compound **98** treatment improved total blood glucose clearance by ~50%.

Charton *et al.* screened a library that led to the identification of S27847 (**99**, Scheme 6) as an AMPK activator.[271] In primary hepatocytes culture and in H-2K muscle cells, **99** was found to be more effective in activating AMPK than the control compound **3**. A focused library of 74 benzimidazole analogs with different modifications on the benzimidazole ring was synthesized and subjected to an AMPK kinase assay using the SAMS peptide as the substrate. This exercise yielded a well-defined SAR around the benzimidazole core and identified analogs with a greater potency.[271] In series A modifications, replacement of 1-phenylcyclohexyl with 1-phenylheptyl (**100**), *trans*-2-phenylcyclopropyl (**101**), biphenyl-2-yl (**102**) showed improved potency compared to the initial hit whereas substitution of cyclohexyl with phenyl (**103**), or phenyl with cyclohexyl (**104**), cyclohexyl with cyclopentyl (**105**), led to a drop in activity. In the second series (B), the phenyl group was conserved and the cyclohexyl group was substituted. Substitution of cyclohexyl with piperidine (**106**) and cyclohexylamine (**107**) resulted in gain of activity whereas substitutions with morpholine (**108**), 4-hydroxypiperidine (**109**), homopiperidine (**110**) resulted in complete loss of activity. In series C, the carbon atom between phenyl and cyclohexyl of **99** was substituted with nitrogen atom (**111**) and no change in activity was observed. On the other hand, substitution of phenylcyclohexylmethyl with 2-(1-piperidino)aniline (**112**) resulted in modest loss of activity. In series D, the phenylcyclohexyl core was kept constant and different substitutions were made at the benzimidazole rings. Substitution of amino at R_1 position (**113**) resulted in similar activity, whereas the nitro group at R_1 (**114**) resulted in complete loss of activity. At R_2 position, substitution of the methyl group (**115**) or methoxy group (**116**) resulted in a gain of activity whereas any other electron withdrawing substitution resulted in a loss of activity. At the present time the exact mechanism of AMPK activation by the benzimidazoles is not known.

ETC-1002 (8-hydroxy-2,2,14,14-tetramethylpentadecanedioic acid) is a novel small molecule currently in clinical trials for the treatment of dyslipidemia and other cardio-metabolic risk factors.[272] ETC-1002 was shown to reduce LDL-cholesterol levels in

preclinical models of dyslipidemia and improve glucose homeostasis in mouse models.[147,273] ETC-1002 has a unique dual mechanism of action. In liver, ETC-1002 inhibits ATP citrate lyase (ACL), a key enzyme in the cholesterol biosynthesis pathway and activates AMPK. HepG2 cells treated with ETC-1002 showed concentration dependent activation of AMPK and ACC phosphorylation that was comparable to metformin. CaMKKβ inhibitor treatment has no effect on ETC-1002-induced AMPK activation suggesting intracellular Ca^{+2}-independent AMPK activation. Also, treatment of ETC-1002 did not alter AMP, ADP and ATP levels suggesting AMPK activation by ETC-1002 is independent of adenine nucleotides. HepG2 cell studies using siRNA showed ETC-1002 activates AMPK in LKB1-dependent fashion.[272]

AMPK Inhibitor

Zhou *et al.* screened a large library and identified a cell permeable pyrazolopyrimidine compound C (**1**, Figure 1) that inhibits the phosphorylation of the SAMS peptide by partially purified AMPK from the liver of SD rats.[68] Kinetic studies using variable ATP concentrations showed compound **1** is a reversible and ATP-competitive inhibitor of AMPK (K_i = 109 ±16 nM) in the absence of **5**. *In vitro* assays using structurally related kinases such as spleen tyrosine kinase (SYK), protein kinase A (PKA), and janus kinase 3 (JAK3) suggested **1** is a selective AMPK inhibitor. Compounds **3** and **60** treatment induces activation of AMPK and inactivation of ACC in primary hepatocytes. This ACC inactivation is inhibited by **1** treatment, which suggests that compound **1** block the stimulation of AMPK activation by pharmacological AMPK activators.

Concluding Remarks

AMPK is a master regulator that controls the energy and metabolic landscape in cells and tissues. There are twelve possible AMPK heterotrimeric complexes that are expressed in cells and tissues. The relative distribution as well as the tissue distribution of AMPK subunits is poorly defined at the present time. Although challenging but development of isoform specific AMPK activators will help elucidate the functional role of AMPK trimeric isoforms. Significant progress has been made towards identification of isoform selective AMPK activators. For instance, Abbott compound **17** is selective for β1-containing AMPK heterotrimers whereas **59** is selective towards α1 and γ1 subunits. AMPK activation can be achieved in different ways such as (i) compounds **17** and **18** bind to the interface between the N-terminal kinase domain and the CBM domain to induce AMPK activation; (ii) **49** binds to charged residues to alter the conformation of the AID to induce AMPK activation; (iii) inhibition of phosphatases etc. There are at least twenty well-characterized substrates of AMPK currently known and additional substrates are being identified through proteomic approaches. Based on available data, it is clear that AMPK is a major signaling hub, however its composition in various cells and tissues is yet to be fully defined. A systematic combination study using different AMPK activators will not only provide useful information regarding the composition of the complexes but also offer opportunities for combination therapy using AMPK activators targeting different subunits/pathways. Additionally studies in various tissues using inhibitors that target upstream kinase and phosphatases to modulate AMPK function irrespective of the composition of the complex could lead to validation of

additional targets. A third and more challenging option is to develop inhibitors against protein-protein interfaces (PPIs) in the AMPK trimeric complex. For example, the structural basis for the regulation of the kinase function by AID has been established. Since the interaction is driven by hydrophobic residues lessons learnt from the development of inhibitors of p53-MDM2/X could used to accelerate this process.[274] In theory, these PPI inhibitors should phenocopy the effects of **49** that binds to charged residues near the AID in the α subunit to activate AMPK. Since the N-terminus of the β subunit undergoes myristoylation that drives the nuclear-cytoplasm shuttling of the AMPK complex, chemical probes against this PPI could help understand the effects of AMPK mislocalization. HTS and peptidomimetic approaches can be used to develop chemical probes that target these PPI's. The availability of AMPK crystal structures offers the possibility of structure-based design of AMPK modulators. *In silico* methods can be used to revisit core structures that have SAR data, which could lead to the identification of suitable compounds for structure-guided optimization. Although most preclinical models suggest that AMPK activators will be useful for the treatment of metabolic diseases, cancer and AD, there are conflicting reports that suggest that AMPK is a contextual oncogene and AMPK inhibition as opposed to activation is beneficial for AD therapy. The importance of AMPK mediated signaling in a plethora of diseases and its complexity suggests that there is an urgent need for additional AMPK modulators that can be used to not only dissect the mechanism of action but also as lead compounds for therapeutic development.

Acknowledgments

The authors would like to acknowledge all the lab members and the reviewers for their valuable suggestions and critiques. The authors are grateful for support from the Nebraska Research Initiative and Fred and Pamela Buffet Cancer Center.

Abbreviations Used

AD	Alzheimer's disease
ADP	Adenosine diphosphate
AICAR	5-Aminoimidazole-4-carboxamide ribonucleotide
AID	Autoinhibitory domain
APP	Amyloid precursor protein
AKT	Protein kinase B
ALA	α-lipoic acid
AMPK	AMP-activated protein kinase
AMP	Adenosine monophosphate
Ara-AMP	Adenine-9-beta-*D*-arabinofuranoside 5′-monophosphate
Aβ	Amyloid-beta protein
AREBP	AICAR response element binding protein
ATP	Adenosine-5′-triphosphate

BRSK1	BR serine/threonine kinase 1
CaMKKβ	Calcium/calmodulin-dependent protein kinase kinase β
CBM	Carbohydrate binding module
CBS	Cystathionine β-synthase
C/EPBα	CCAAT/enhancer binding protein α
dAMP	2′-Deoxy-AMP, 2′-deoxyadenosine-5′-monophosphate
DNL	*De novo* lipogenesis
2-DG	2-deoxyglucose
DIO	Diet-induced obese
DMC	Dimethoxycurcumin
dhBBR	dihydroberberine
DR	Death receptor
eEF	Elongation factors
EGCG	Epigallocatechin-3-gallate
EGFR	Epidermal growth factor receptor
ERK	Extracellular signal-regulated kinases
ER	Endoplasmic reticulum
eNOS	Endothelial nitric-oxide synthase
4EBP1	Eukaryotic initiation factor 4E binding protein 1
FBPase	Fructose-1,6-bisphosphatase
FRET	Fluorescence resonance energy transfer
GBD	Glycogen binding domain
GPPase	Glycogen phosphorylase
GSK-3β	Glycogen synthase kinase-3-beta
GLUT	Glucose transporters
GS	Glycogen synthase
GSK	GlaxoSmithKline
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
HNF4α	Hepatocyte nuclear factor 4-alpha
HTS	High throughput screening
IL-6	Interleukin 6
IR	insulin receptor

IRS-1	Insulin receptor substrate
JAK3	Janus kinase 3
JNK	c-Jun N-terminal kinases
KD	Kinase domain
LDH	Lactate dehydrogenase
LKB1	Liver Kinase B1
LPS	Lipopolysaccharide
LXR	Liver X receptors
MEFs	Mouse embryonic fibroblasts
MARK2	MAP/microtubule affinity-regulating kinase 2
MELK	Maternal embryonic leucine zipper kinase
mTOR	Mammalian target of rapamycin
mTORC1	Mammalian target of rapamycin complex-1
MODY	Maturity onset diabetes of the young
MRLC	Myosin regulatory light chain
NADH	Nicotinamide adenine dinucleotide
NFTs	Neurofibrillary tangles
NO	Nitric Oxide
NOS	Nitric oxide synthase
NUAK2	NUAK family SNF1-like kinase 2
OCT	Organic cation transporters
PAN	Panduratin
PKA	Protein kinase A
PPARγ	Peroxisome proliferator-activated receptor-γ
PFK	Phosphofructokinase
PEPCK	Phosphoenolpyruvate carboxykinase
PI3K	Phosphatidylinositide 3-kinases
PFK-2	phosphofructokinase-2
Ppm1E	Protein serine/threonine phosphatase 1E
PPP1R12C	protein phosphatase 1 regulatory subunit 12C
PAK2	p21-activated protein kinase
P-gp	P-glycoprotein

PIKfyve	FYVE finger-containing phosphoinositide kinase
PtdIns	phosphatidylinositides
PJS	Peutz-Jeghers syndrome
PPI	Protein-protein interfaces
SAR	Structure activity relationship
S-AMP	adenyl-succinic acid
SAICAR	5-Amino-4-imidazole-*N*-succinocarboxamide ribonucleotide
6-SH-PMP	6-mercaptopurine riboside 5′-monophosphate
S6K	Ribosomal S6 kinase
SD	Sprague-Dawley
SH2	Src homology 2
SREBP	Sterol regulatory element binding protein
SIRT1	Silent mating type information regulation 2 homolog 1
SYK	Spleen tyrosine kinase
TAK1	Transforming growth factor-β activated protein kinase-1
TCA	Tricarboxylic acid
TSC	Tuberous sclerosis complex
TZDs	Thiazolidinediones
TMPA	Ethyl 2-[2,3,4-trimethoxy-6-(1-octanoyl)phenyl]acetate
TRAIL	Tumor necrosis factor-related apoptotic-inducing ligand
Tu-MP	tubercidin 5′-monophosphate
ZMP	5-amino-4-imidazolecarboxamide ribotide

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Biographies

Sandeep Rana is a Research Assistant Professor at the Eppley Institute for Cancer Research and Allied Diseases, University of Nebraska Medical Center. Prior to joining UNMC, he completed a two-year postdoctoral research position at Mayo Clinic, MN. He received his Master's degree in chemistry from University of Delhi, India. After finishing his master's, he was awarded a research fellowship from the Council of Scientific and Industrial research, New Delhi to pursue his graduate studies. He moved to the United States, attended Kansas State University and received his Ph.D. in organic chemistry under the guidance of Dr. Duy H Hua in 2009. His research interests lie in medicinal chemistry and drug discovery in the area of cancer and neurodegenerative diseases.

Elizabeth C. Blowers is a graduate student at the Eppley Institute for Cancer Research and Allied Diseases, University of Nebraska Medical Center. She completed her bachelor's degree in biology at the University of Tennessee, where she studied in the laboratory of Dr. Erik Zinser. Her research at UNMC focuses on the characterization of small molecule inhibitors that target posttranslationally modified forms of proteins implicated in disease.

Amarnath Natarajan, Professor, Eppley Institute for Research in Cancer and Allied Diseases, University of Nebraska Medical Center. Dr. Natarajan hails from the south Indian state of Tamil Nadu. He completed his bachelors and masters in chemistry from Madras Christian College and Indian Institute of Technology, Chennai, respectively. He obtained his PhD in organic chemistry from the University of Vermont and conducted postdoctoral research at Harvard Medical School. He started his independent career as an Assistant Professor at the University of Texas Medical Branch. His lab is focused on developing chemical probes to dissect signaling pathways relevant to human diseases.

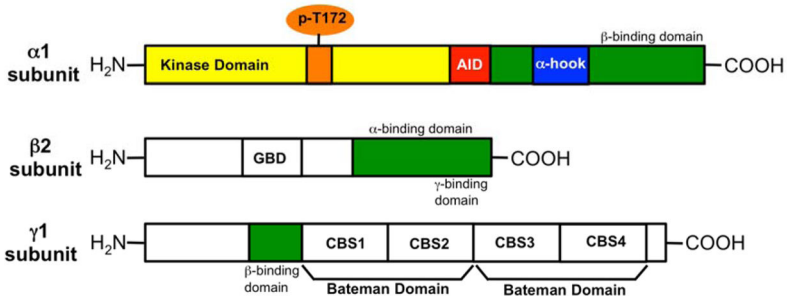


Figure 1.
Domain architecture of α1, β2 and γ1 AMPK subunits.

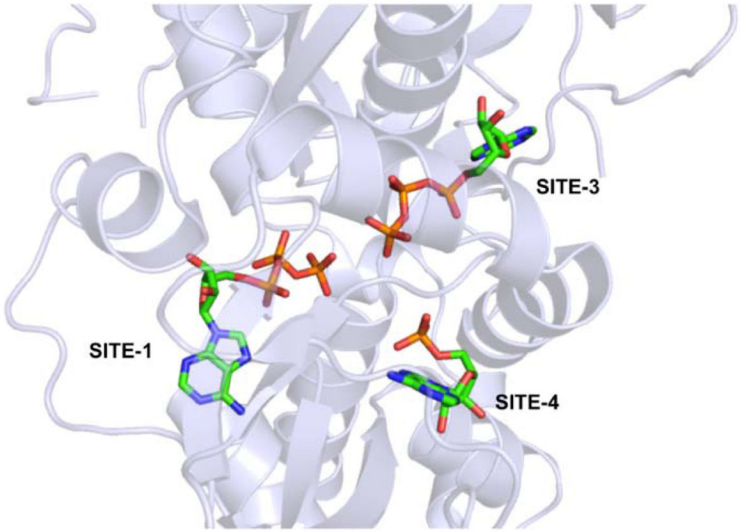


Figure 2.
Adenine nucleotide binding sites on the γ subunit of AMPK (generated using coordinates from PDB code 2V92 using pymol).

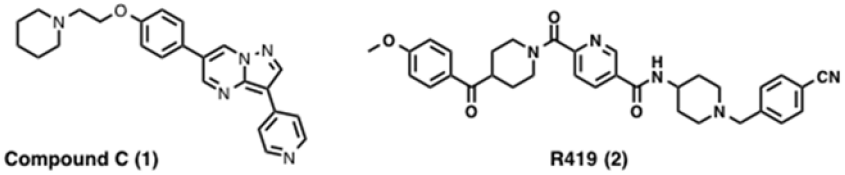


Figure 3.
Structure of compound C and R419.

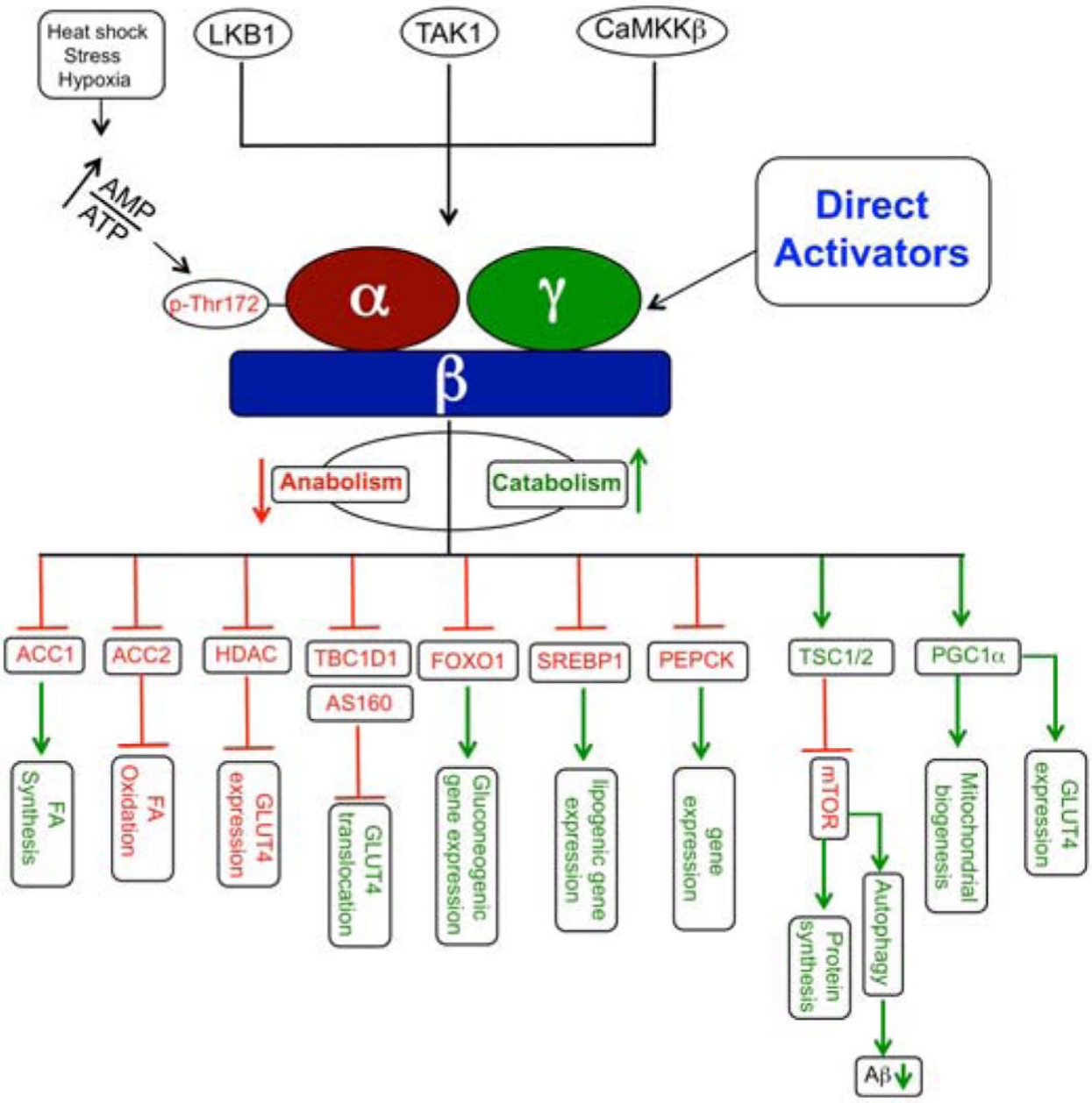


Figure 4.
AMPK signaling pathways and their downstream effects.

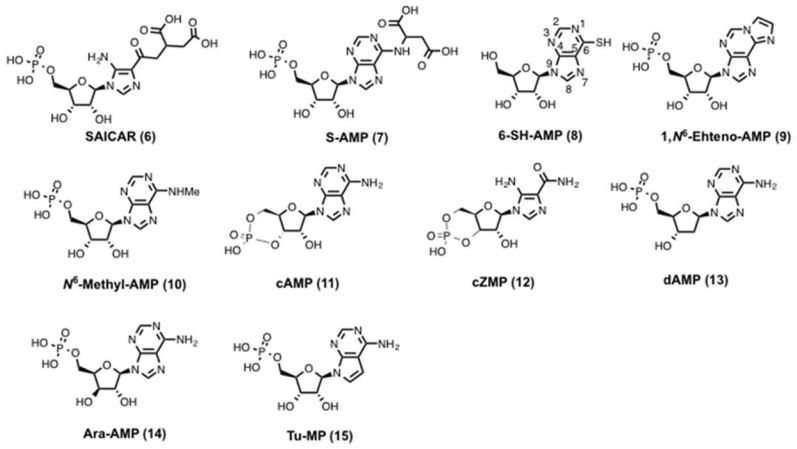


Figure 5.
Structure of AMP analogs.

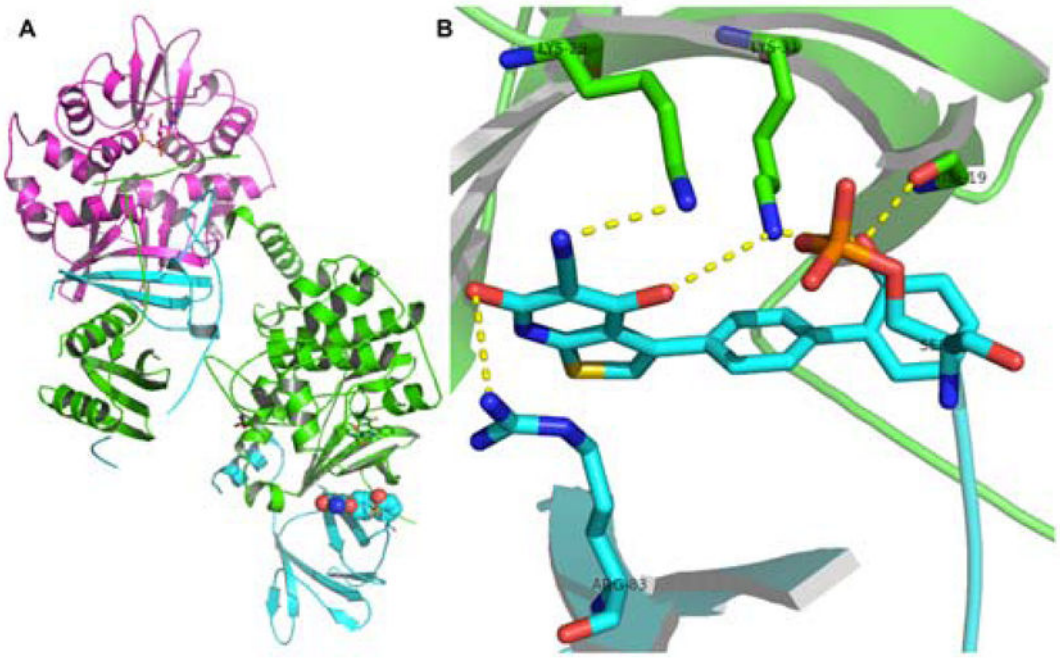


Figure 6.

(A) Cartoon representation of full-length α2β1γ1 in complex with **17**, represented in spheres. (B) Polar interactions that contribute to activator binding.

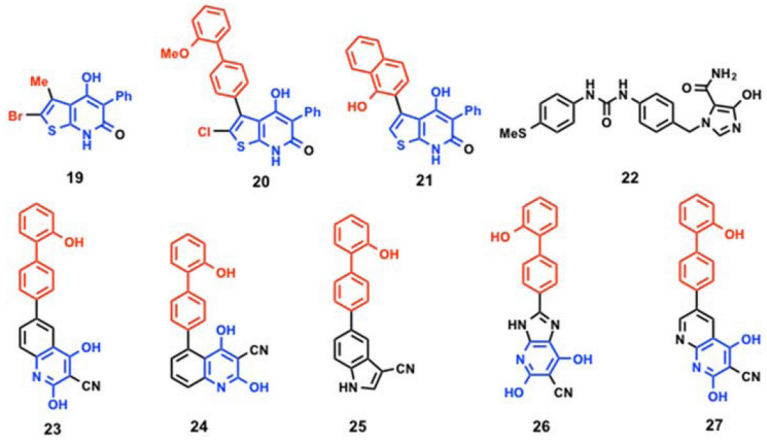


Figure 7.
Direct AMPK activators.

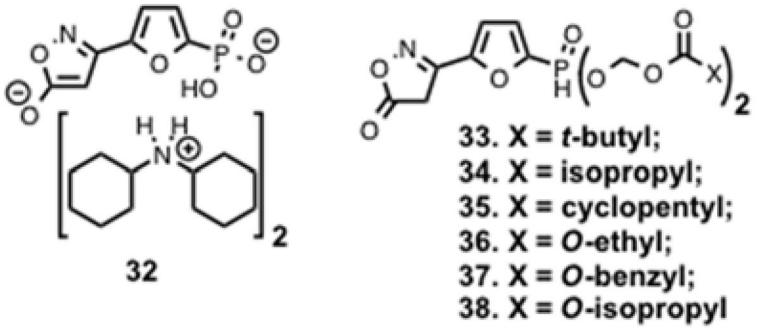
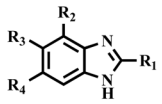


Figure 8.
AMPK activators that inhibit DNL.



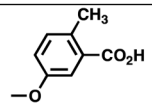
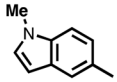
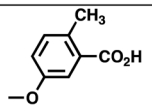
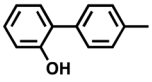
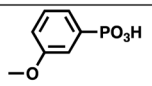
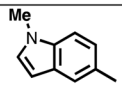
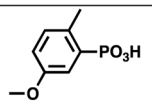
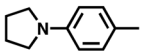
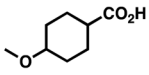
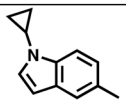
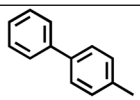
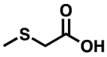
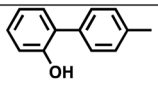
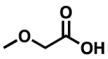
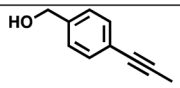
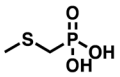
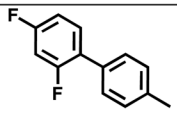
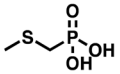
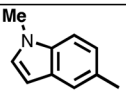
S. no	EC_{50} (nM)	R_1	R_2	R_3	R_4	Act_{max}
39	3.0		H		Cl	241%
40	1.0		H		Cl	189%
41	0.6		H		Cl	173%
42	1.1		H		Cl	223%
43	4		F		F	504%
44	17		F		F	631%
45	13		H		Cl	150%
46	97		H		Cl	157%
47	184		H		Cl	249%
48	58		H		Cl	168%

Figure 9.
General core structures of benzimidazole AMPK activators.

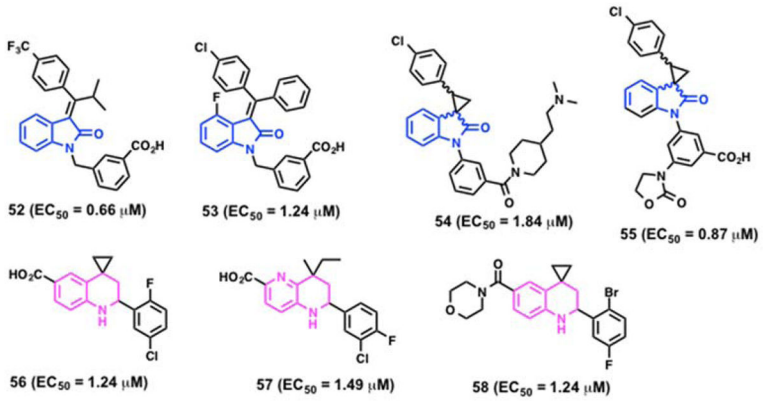
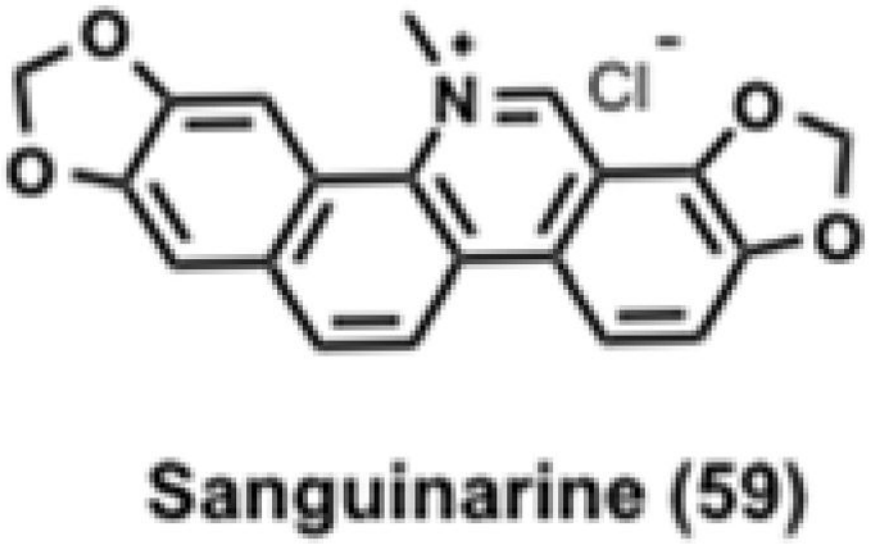


Figure 10.
AMPK activators reported by Hoffmann-La Roche AG.



Sanguinarine (59)

Figure 11.
Structure of Sanguinarine, an AMPK activator.

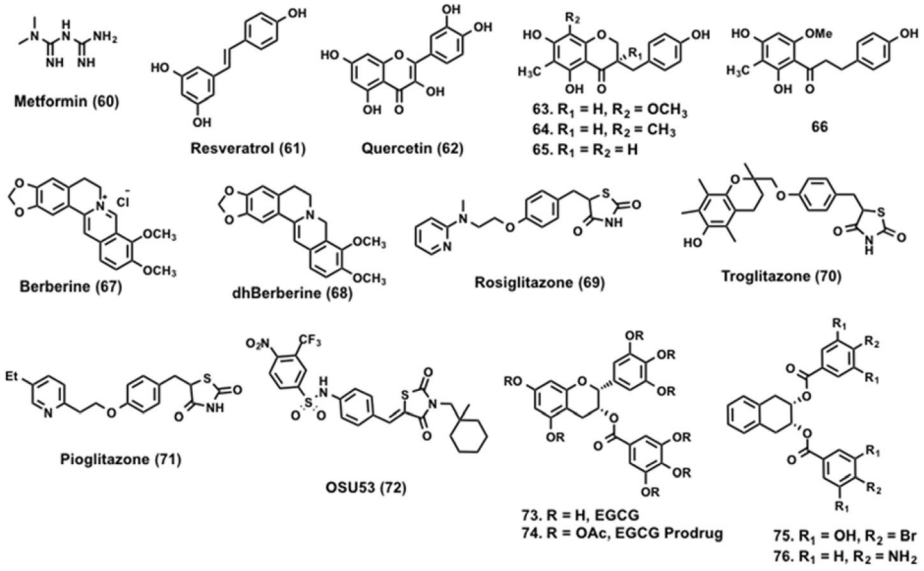


Figure 12.
Indirect AMPK activators.

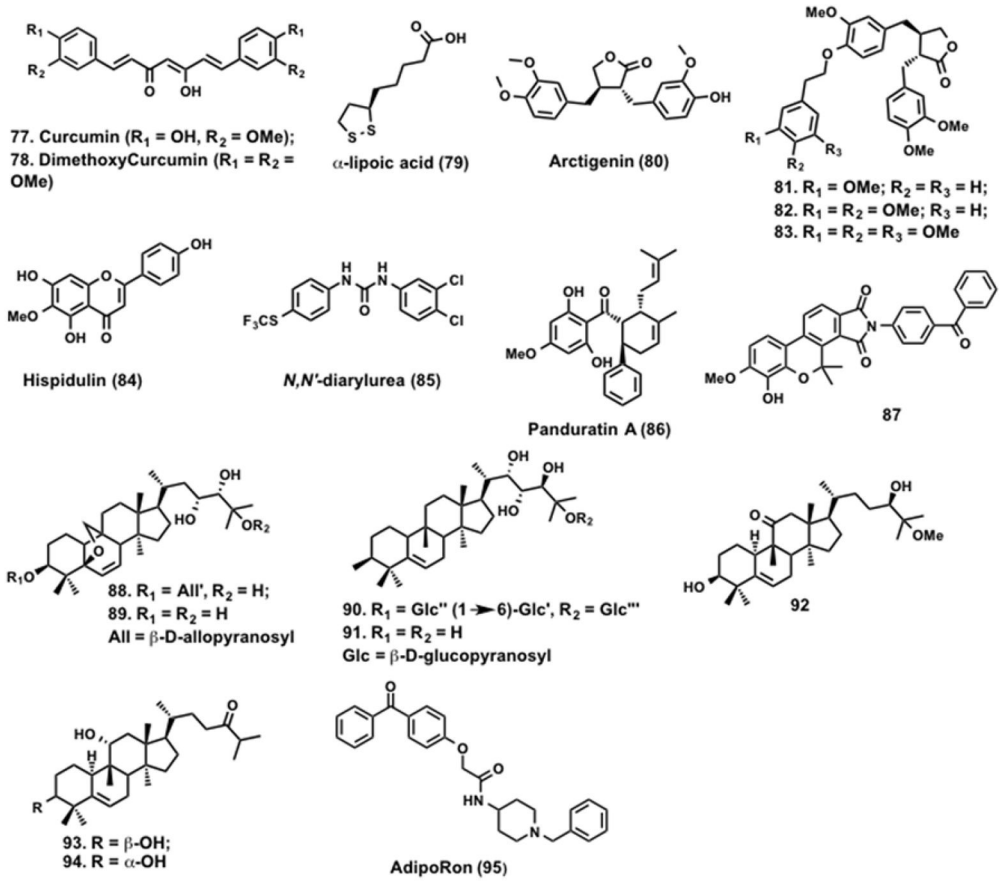
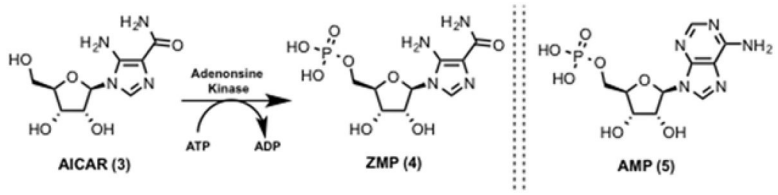
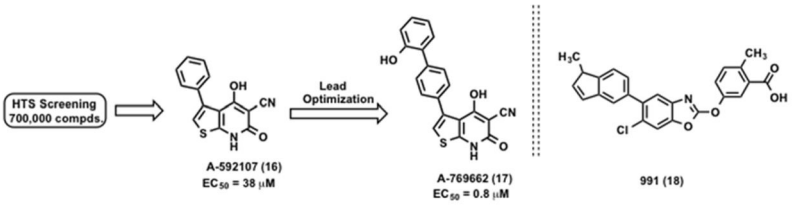


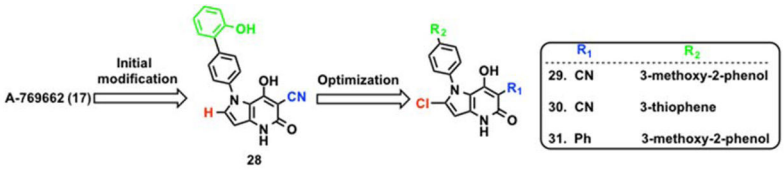
Figure 13.
Indirect AMPK activators.



Scheme 1.
Phosphorylation of AICAR and structure of AMP.

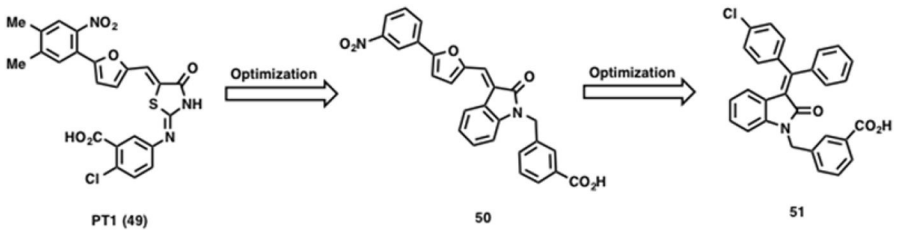


Scheme 2.
Identification and optimization of thienopyridone compounds.



Scheme 3.

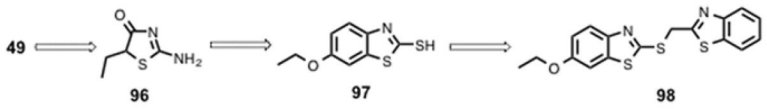
Initial modification and optimization of pyrrolopyridone analogs



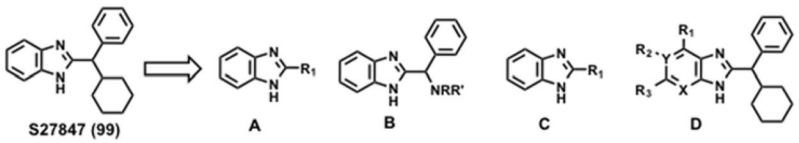
PT1 (49) 50 51

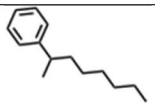
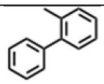
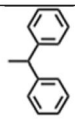
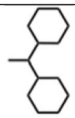
Scheme 4.

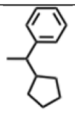
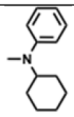
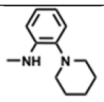
Structure of initial hit PT1 (**49**) and other optimized AMPK activators.



Scheme 5.
Optimization of benzothiazole AMPK activators.



Cpd. no	Series	Substitutions	Rel. enzyme activity (100 μM)
S27847			4.5
100	A	R_1 = 	7.1
101	A	R_1 = 	5.4
102	A	R_1 = 	5.1
103	A	R_1 = 	1.1
104	A	R_1 = 	1.1

105	A	R_1 = 	0.8
106	B	NRR' = 	6.1
107	B	NRR' = 	5.8
108	B	NRR' = 	1.0
108	B	NRR' = 	1.2
110	B	NRR' = 	1.0
111	C	R_1= 	4.3
112	C	R_1= 	3.1
113	D	$R_1 = NH_2$; R_2 = H; R_3 = H; X = CH; Y = C.	4.3
114	D	$R_1 = NO_2$; R_2 = H; R_3 = H; X = CH; Y = C.	1.0
115	D	R_1 = H; R_2 = Me; R_3 = H; X = CH; Y = C.	6.9
116	D	R_1 = H; R_2 = OMe; R_3 = Me; X = CH; Y = C.	5.0

Scheme 6.
Identification of S27847 and its novel series.

Table 1

AMPK direct substrates and their phosphorylation sites (those reviewed here are highlighted in bold).

S. No	Substrate	Residue
1	Acetyl-CoA carboxylase 1[5]	Ser-80, Ser-1201 and Ser-1216
2	Acetyl-CoA carboxylase 2[7]	Ser-221 and other minor phosphorylation site
3	Glycogen synthase (muscle)[3]	Ser-8
4	HMG-CoA reductase[3]	Ser-872
5	**Zinc finger transcription factor (AREBP)**[8]	Ser-470
6	**Eukaryotic elongation factor (eEF2) kinase**[4]	Ser-398
7	**Endothelial NO synthase (eNOS)**[10]	Ser-1177 (in presence of Ca^{+2}/calmodulin) and Thr-495 (in absence of Ca^{+2}/calmodulin)
8	**GLUT4 enhancer factor (GEF)**[58]	-
9	**Hepatic nuclear factor (HNF4α)**[12]	Ser-304 and other minor phosphorylation site
10	**Insulin receptor substrate-1**[13]	Ser-789
11	**$p27^{Kip1}$**[15]	Thr-198
12	**Transcriptional coactivator p300**[16]	Ser-89
13	**6-phosphofructo-2-kinase (PFK-2)**[17]	Ser-466 and Ser-483
14	**Tuberous sclerosis 2 (TSC2)**[18]	Thr-1227 and Ser-1345
15	**Protein phosphatase 1 regulatory subunit 12C (PPP1R12C)**[19]	Ser-452
16	**p21-activated protein kinase (PAK2)**[19]	Ser-20
17	**Cardiac troponin I (cTnI)**[59]	Ser-150
18	**PIKfyve (FYVE domain-containing phosphatidylinositol 3-phosphate 5-kinase)**[60]	Ser-307

Table 2

AMPK heterotrimers activity profile with compound **29**.

AMPK Isoforms	Activity (pEC_{200})	AMPK Isoforms	Activity (pEC_{200})
α1β1γ1	9.2	α1β2γ1	6.3
α1β1γ2	9.4	α2β2γ1	73
α1β1γ3	8.5	α2β2γ3	7.3
α2β1γ1	8.4		

pEC_{200} = −log(compound concentration leading to a 2-fold AMPK activity increase)

Table 3

In vitro and *in vivo* inhibition of DNL.

Compound no	**Rat EC_{50} (nM)**	***In vivo* DNL inhibition (%)**
32	> 10000	Not determined
33	100	65
34	20	78
35	30	Not determined
36	42	73
37	609	34
38	27	73