

REVIEW

AMPK activators: mechanisms of action and physiological activities

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AMP-activated protein kinase (AMPK) is a central regulator of energy homeostasis, which coordinates metabolic pathways and thus balances nutrient supply with energy demand. Because of the favorable physiological outcomes of AMPK activation on metabolism, AMPK has been considered to be an important therapeutic target for controlling human diseases including metabolic syndrome and cancer. Thus, activators of AMPK may have potential as novel therapeutics for these diseases. In this review, we provide a comprehensive summary of both indirect and direct AMPK activators and their modes of action in relation to the structure of AMPK. We discuss the functional differences among isoform-specific AMPK complexes and their significance regarding the development of novel AMPK activators and the potential for combining different AMPK activators in the treatment of human disease.

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INTRODUCTION

As a cellular energy sensor, AMP-activated protein kinase (AMPK) is activated in response to a variety of conditions that deplete cellular energy levels, such as nutrient starvation (especially glucose), hypoxia and exposure to toxins that inhibit the mitochondrial respiratory chain complex.^{1,2} AMPK is a serine/threonine protein kinase complex consisting of a catalytic α -subunit ($\alpha 1$ and $\alpha 2$), a scaffolding β -subunit ($\beta 1$ and $\beta 2$) and a regulatory γ -subunit ($\gamma 1$, $\gamma 2$ and $\gamma 3$; Figure 1). Ubiquitous expression of AMPK $\alpha 1$ -, $\beta 1$ - and $\gamma 1$ -subunits in many tissues makes the $\alpha 1\beta 1\gamma 1$ complex a reference for AMPK assays to identify AMPK activators. However, given the unique functions and/or subcellular (or tissue)-specific distribution of the distinct AMPK complex,^{3–5} referencing screening to the $\alpha 1\beta 1\gamma 1$ complex may present a limited range of the physiology of AMPK. In line with this notion, increasing evidence shows that inactivating mutations and genetic deletion of specific isoforms produce tissue-specific physiological results.^{6–8} Mutations in the AMPK $\gamma 2$ subunit have frequently been observed in human cardiomyopathies, and deletion of the AMPK $\alpha 2$ subunit, but not $\alpha 1$, has been shown to decrease infarct volume in mouse models of stroke.

Allosteric activation of AMPK by AMP

The first class of direct AMPK activators is small molecules that mimic cellular AMP. These molecules trigger a conformational

change in the AMPK complex that allows further activation by phosphorylation of Thr-172 in the AMPK α subunit.^{9,10} The molecular mechanism underlying allosteric activation of AMPK by AMP binding has been demonstrated by several recent studies of the three-dimensional structure of AMPK.^{11–13} This crystal structure has shown the importance of cystathionine- β -synthase domain repeats within the AMPK γ subunit in the molecular mechanism by which AMPK is activated in response to cellular adenosine nucleotides (AMP, ADP or ATP). Four consecutive cystathionine- β -synthase domains in the AMPK γ subunit provide four potential adenine nucleotide-binding sites. These sites are numbered Sites 1–4, according to the number of the cystathionine- β -synthase domain repeat carrying a conserved aspartate residue involved in ligand binding.^{11,14,15} In the mammalian AMPK $\gamma 1$ subunit, Site 2 appears to be always empty and Site 4 to have a tightly bound AMP molecule, whereas Sites 1 and 3 represent the regulatory sites that bind AMP, ADP or ATP, which compete for binding.¹⁶ AMP binding to Site 1 appears to cause allosteric activation, whereas binding of AMP or ADP to Site 3 appears to modulate the phosphorylation state of Thr172.¹³ Although cellular ADP levels are higher than those of AMP, a recent study has shown that AMP is a bona fide activator that enhances LKB1-dependent Thr 172 phosphorylation *in vivo*.¹⁷ AMP binding to the AMPK γ subunit serves as an important regulatory feature of the conformational switch that activates

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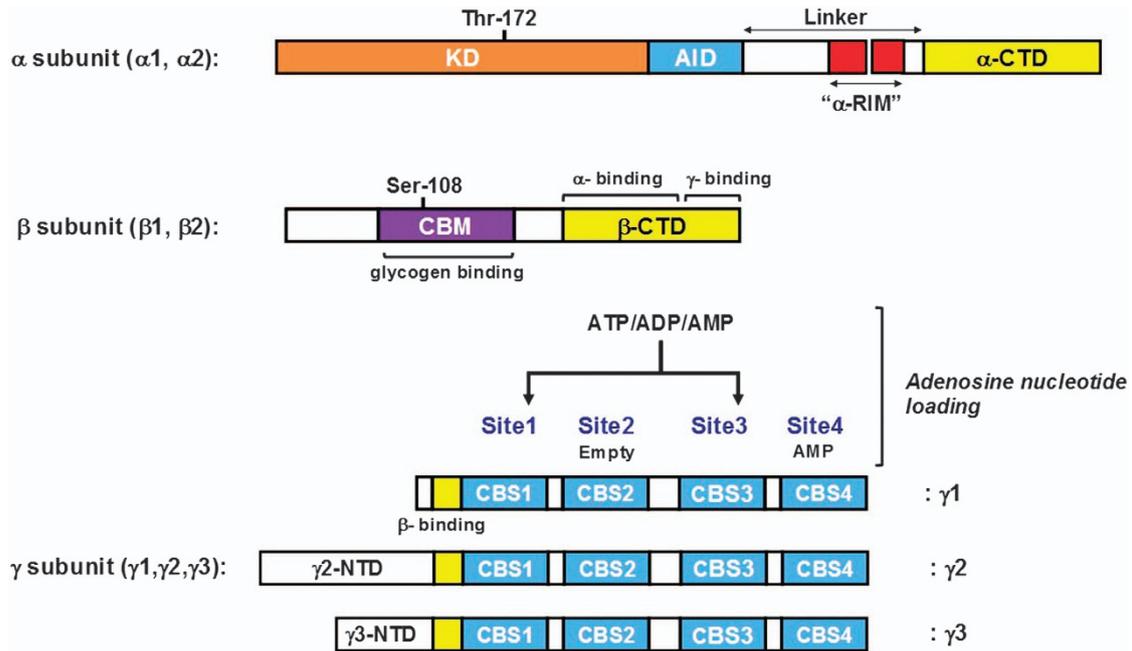


Figure 1 Functional domains of AMP-activated protein kinase (AMPK) subunits. The mammalian $\alpha 1/\alpha 2$ and $\beta 1/\beta 2$ isoforms are very similar, and their characteristic features are shown. AMPK α subunits: KD, kinase domain containing Thr-172 for the activation by upstream kinases; AID, autoinhibitory domain; two α -RIM, regulatory subunit interacting motifs triggering the conformational changes in response to AMP binding to the AMPK γ subunit; α -CTD, C-terminal domain binding to the β -subunit. AMPK β subunit: CBM, carbohydrate-binding module, in which Ser108 is important for the action of some direct AMPK activators, such as thienopyridone (A-769662) and salicylate; β -CTD, C-terminal domain containing α -subunit-binding site and immediately followed by the domain for γ -subunit interaction. AMPK γ subunit: three γ -subunit isoforms have variable N-terminal domains (NTDs); four CBS, cystathionine- β -synthases domain, which forms two Bateman domains that create four adenosine nucleotide-binding sites (Sites 1–4). Site 2 appears to be always empty and Site 4 to have a tightly bound AMP, whereas Sites 1 and 3 represent the regulatory sites that bind AMP, ADP or ATP in competition.

the AMPK complex. The catalytic AMPK α subunit contains an N-terminal kinase domain (KD) immediately followed by an autoinhibitory domain (AID). The three-dimensional structure shows that the AID interacts with the small and large lobes of the KD and causes AMPK to be maintained in an inactive conformation. Once AMP binds to the AMPK γ subunit, the α -RIM (regulatory subunit-interacting motif) between the KD/AID and a globular C-terminal domain of the AMPK α subunit interact with one of the regulatory adenosine nucleotides on the AMPK γ subunit in a manner akin to two arms wrapping around the adenosine. These conformational changes release and expose the KD of AMPK α from its AID to activate the AMPK complex.

Regulation of AMPK activity by upstream kinases

Physiological AMPK activation involves phosphorylation of Thr-172 within the activation loop of the KD in the AMPK α catalytic subunit. Two upstream kinases, LKB1¹⁸ and CaMKK β (Ca²⁺/calmodulin-dependent protein kinase β),¹⁹ have been extensively documented to phosphorylate Thr-172 of the AMPK α subunit. Notably, there are lines of evidence showing that the LKB1-dependent AMPK α phosphorylation at Thr172 is greatly enhanced by the binding of AMP to the AMPK γ -subunit, and, at the same time, the AMP-binding inhibits dephosphorylation of this activating phosphorylation by protein phosphatases, such as PP2A and PP2C *in vitro*.^{20,21}

Interestingly, the effect of AMP on Thr172 phosphorylation of the AMPK α -subunit appears to be dependent on N-terminal myristoylation of the β -subunit, although the underlying mechanism remains to be demonstrated.²² In contrast to the LKB1 complex, another upstream AMPK kinase, CaMKK β , can activate AMPK in response to increases in cellular Ca²⁺ without any significant change in ATP/ADP/AMP levels. Treatments that deplete cellular ATP do not effectively activate AMPK in LKB1-negative tumors because the basal activity of CaMKK β is too low to affect the phosphorylation status of AMPK α Thr172, although the increase in AMP due to ATP depletion makes the AMPK α -subunit a better substrate for CaMKK β . However, these treatments can cause AMPK activation under conditions that elevate intracellular Ca²⁺. These data indicate that the phosphorylation/dephosphorylation equilibrium at Thr-172 on the AMPK α -subunit involves AMP binding to the AMPK γ subunit and N-terminal modification of the AMPK β -subunit, adding another a level of complexity to the AMPK activation mechanism.

Physiological functions of AMPK

As its name suggests, AMPK has a key role in maintaining the balance between anabolic and catabolic programs for cellular homeostasis in response to metabolic stress.^{23–28} Given the functional attributes of AMPK in glucose/lipid homeostasis, body weight, food intake, insulin signaling and mitochondrial

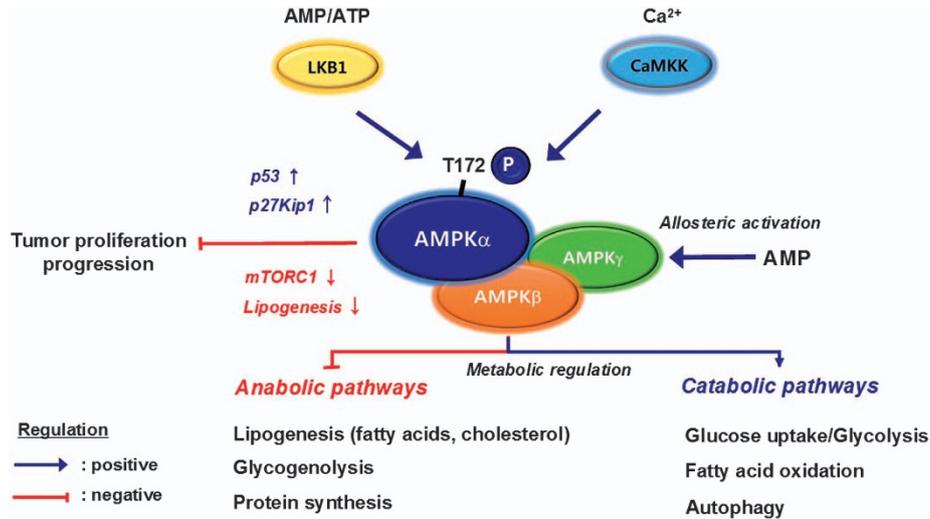


Figure 2 A summary of the physiological roles of AMP-activated protein kinase (AMPK).

biogenesis, AMPK is considered to be a major therapeutic target for the treatment of metabolic diseases including type 2 diabetes and obesity.^{29,30}

A number of studies have shed light on the role of AMPK in tumorigenesis.³¹ An initial report connecting AMPK to cancer biology described the discovery of the tumor suppressor LKB1 as a major AMPK upstream kinase.³² Genetic mutations of the *LKB1* gene are responsible for inherited Peutz-Jeghers syndrome, which is characterized by the development of hamartomatous polyps in the intestine.³³ Since then, a number of *in vitro* and *in vivo* studies have suggested that AMPK indeed mediates the tumor-suppressor effects of LKB1. This is supported by findings that drugs that are capable of activating AMPK (metformin, phenformin, A-769662) delay the onset of tumorigenesis in *in vivo* models.^{34,35} Much effort has been made to understand the molecular mechanisms underlying the antitumorigenic functions of AMPK. These studies have shown that mTORC1^{36,37} and RNA polymerase I transcription factor TIF-1A,³⁸ both of which are required for rapidly proliferating cells, are under the control of AMPK. In addition, AMPK activation has been shown to cause G1 cell cycle arrest, which is associated with activation of p53, followed by induction of the cell cycle inhibitor protein, p21.^{39,40} Similarly, AMPK has been shown to cause cell cycle arrest by inducing the phosphorylation and concomitant stabilization of the cyclin-dependent kinase inhibitor p27^{Kip1} in response to metabolic stress.⁴¹ A recent study has described an additional layer of p53–AMPK–mTORC1 regulation via the p53-responsive gene products Sestrin1/2.⁴² However, it should be noted that AMPK might protect tumor cells against the action of cytotoxic agents, nutrient limitation and hypoxia, once the tumors are established. Therefore, AMPK activators might be deleterious in the treatment of cancer.

Another important aspect of AMPK biology is the role of AMPK in autophagy, a lysosome-dependent catabolic program that maintains cellular homeostasis.^{43–46} A number of studies

have demonstrated that AMPK has important roles in autophagy regulation by directly phosphorylating two autophagy-initiating regulators: a protein kinase complex ULK1 (Unc-51-like autophagy-activating kinase)^{47,48} and a lipid kinase complex PI3KC3/VPS34 (phosphatidylinositol 3-kinase, catalytic subunit type 3; also known as VPS34).⁴⁹ A number of reports have demonstrated the metabolic significance of autophagy in glycogenolysis (glycophagy)⁵⁰ and lipolysis (lipophagy)⁵¹ and even in regulating adipose mass as well as differentiation *in vivo*.⁵² In this regard, elucidating the molecular connection between AMPK and autophagy will provide a novel avenue to expand the functional network of AMPK in cellular homeostasis, including metabolism.

Given these functional attributes, as summarized in Figure 2, much effort has been made to develop robust AMPK assays and to identify AMPK modulators to provide therapies for a variety of human diseases.^{53–56} In this review, we present a comprehensive summary of both indirect and direct AMPK activators and their modes of action in relation to the structure of AMPK, and discuss the implications of AMPK as a therapeutic target.

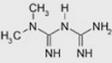
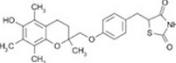
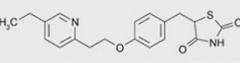
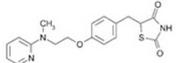
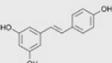
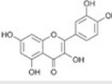
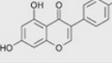
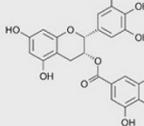
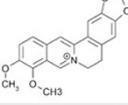
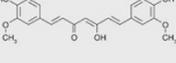
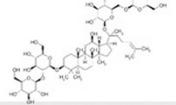
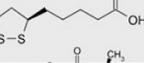
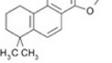
INDIRECT AMPK ACTIVATORS

Practically, AMPK can be activated by any modulator that causes AMP or calcium accumulation. These are classified as indirect activators because a direct interaction between AMPK and modulators is not necessary. Indirect AMPK activators are listed on Table 1.

Biguanides

Metformin is a type of biguanide, a synthetic derivative of guanide that is a natural product from the plant *Galega officinalis*, and has been used as a first-line antidiabetic drug because of its ability to reduce hepatic glucose production and enhance peripheral insulin sensitivity.⁵⁷ A number of studies have demonstrated that the actions of metformin are

Table 1 Indirect AMPK activators

Compound name	Chemical structure	Activation mechanism	Molecular target	reference
Metformin		increase of AMP:ATP ratio	Complex I of the mitochondrial respiratory chain	58-62
Troglitazone		increase of AMP:ATP ratio	Complex I of the mitochondrial respiratory chain	63-65
Pioglitazone		increase of AMP:ATP ratio	Complex I of the mitochondrial respiratory chain	63-65
Rosiglitazone		increase of AMP:ATP ratio	Complex I of the mitochondrial respiratory chain	63-65
Resveratrol		increase of AMP:ATP ratio	the mitochondrial F ₁ F ₀ -ATPase/ATP synthase	66,67,72,73
Quercetin		increase of AMP:ATP ratio	the mitochondrial F ₁ F ₀ -ATPase/ATP synthase	68,72,73
Genistein		increase of AMP:ATP ratio	the mitochondrial F ₁ F ₀ -ATPase/ATP synthase	69,72,73
Epigallocatechin gallate		increase of AMP:ATP ratio	the mitochondrial F ₁ F ₀ -ATPase/ATP synthase	69,72,73
Berberine		increase of AMP:ATP ratio	Complex I of the mitochondrial respiratory chain	70,74
Curcumin		increase of AMP:ATP ratio	the mitochondrial F ₁ F ₀ -ATPase/ATP synthase	71-73
Ginsenoside Rb1		increase of AMP:ATP ratio	unknown	75,76
α-lipoic acid		increase of cellular calcium level	unknown	77-82
Cryptotanshinone		increase of ROS	unknown	87,88

attributable to AMPK. Zhou *et al.* have revealed the molecular mechanisms by which AMPK mediates the antidiabetic actions of metformin: stimulation of fatty-acid oxidation and glucose uptake, and downregulation of lipogenic genes and hepatic glucose production.⁵⁸ AMPK activation by metformin is not a result of direct activation; instead, metformin inhibits complex I of the mitochondrial respiratory chain, leading to an increased AMP:ATP ratio.⁵⁹ This indirect mechanism has further been supported by the observation that metformin fails to activate AMPK in cells expressing the AMP-insensitive

(R531G) AMPK γ 2 subunit.⁶⁰ Recent findings by Fullerton *et al.* have also shown that phosphorylation of acetyl-CoA carboxylase by AMPK is required for the lipid-lowering effect and the insulin-sensitizing effects of metformin, thereby supporting the role of AMPK in metformin action. However, the role of AMPK has been called into question by recent work showing that metformin lowers blood glucose levels in animal models of liver-specific AMPK α knockout or LKB1 knockout.⁶¹ Thus, further studies are required to distinguish the AMPK-dependent and -independent effects of metformin.

Table 2 Direct AMPK activators

Compound name	Chemical structure	Target subunit	Isoform specificity	reference
5-Aminoimidazole-4-carboxamide riboside (AICAR)		AMPK γ subunit (AMP-mimetic)	none	91,92
Thienopyridone (A-769662)		AMPK β subunit	β 1- subunit	98-102
Benzimidazole (Compound 911)		AMPK β subunit	β 1- subunit	12
Salicylate (Pro-drug of Aspirin)		AMPK β subunit	β 1- subunit	103
Compound-13 (Pro-drug of C-2)		AMPK α subunit	α 1- subunit	108,109
PT-1		AMPK β subunit	γ 1- subunit	111,112
MT 63-78 (Debio0930)		AMPK β subunit	β 1- subunit	113

Thiazolidinedione

Thiazolidinediones (TZDs), also known as glitazones, are a class of insulin-sensitizing drugs including troglitazone, pioglitazone and rosiglitazone. TZDs act primarily by activating the nuclear hormone receptor peroxisome proliferator-activated receptors (PPARs), notably PPAR γ , for which their affinity is highest. They are also known to exert their antidiabetic effect in part through AMPK activation. TZDs rapidly activate AMPK in a variety of tissues including skeletal muscle,^{62,63} liver and adipose tissue,⁶⁴ and the activation mechanisms are associated with accumulation of AMP as a result of inhibiting complex I of the mitochondrial respiratory chain.⁶⁵ In addition, TZD treatment induces the expression and release of adiponectin from adipocytes,⁶³ which in turn activates AMPK in skeletal muscle and the liver, resulting in increased glucose uptake and fatty-acid oxidation, and decreased hepatic glucose production. Thus, AMPK can be activated by TZDs through at least two different mechanisms.

Polyphenols

In addition to pharmaceutical agents, numerous naturally occurring compounds and phytochemicals have been shown to activate AMPK. Among them are polyphenols, a structural class of natural or synthetic products characterized by the

presence of multiples of phenol structure units. Despite the structural variance, numerous polyphenols are capable of activating AMPK, and they exert beneficial effects on type 2 diabetes and metabolic syndrome. These include resveratrol from red grapes,^{66,67} quercetin from many plant units including fruits, vegetables and grains,⁶⁸ genistein found in a number of plants such as soybeans,⁶⁹ epigallocatechin gallate from green tea,⁶⁹ berberine from *Coptis chinensis*⁷⁰ and curcumin from *Curcuma longa*.⁷¹ Mechanisms of activation of AMPK by these compounds appear to require the elevation of AMP levels because many of these compounds are known to inhibit mitochondrial ATP production. Resveratrol, quercetin, epigallocatechin-3-gallate and curcumin target and inhibit the mitochondrial F₁F₀-ATPase/ATP synthase,^{72,73} whereas berberine is associated with the inhibition of respiratory chain complex I.⁷⁴ The molecular mechanism of AMPK activation by resveratrol, berberine and quercetin has further been supported by the observation that these compounds fail to activate AMPK in cells expressing AMP-insensitive (R531G) AMPK γ 2 subunit.⁶⁰

Ginsenoside

Panax ginseng has been long known to have favorable effects in type 2 diabetes and metabolic syndrome. Ginsenosides,

a class of tetracyclic triterpene glycosides, are the major pharmacological ingredients in ginseng. To date, more than 80 structurally different ginsenosides have been isolated from the plant genus *Panax*, and a number of ginsenosides, including Rb1, Rb2, Rc, Re, Rg1, Rg2 and Rg3, have been reported to activate AMPK, resulting in an increased glucose uptake, decreased hepatic triglyceride and cholesterol levels, and the inhibition of lipogenesis and hepatic glucose production.⁷⁵ The mechanisms for AMPK activation by ginsenosides are largely unknown; however, presumably these compounds are likely to activate AMPK via AMP-dependent mechanisms because the ginsenoside, Rb1, has been reported to increase the intracellular AMP:ATP ratio.⁷⁶

α -Lipoic acid

α -Lipoic acid (ALA), a naturally occurring dithiol compound derived from octanoic acid, has a critical role in mitochondrial bioenergetics reactions by acting as a cofactor for pyruvate dehydrogenase and α -ketoglutarate dehydrogenase. Owing to its powerful antioxidant property, ALA has gained substantial attention for use in managing diabetic complications.⁷⁷ Recent studies have also demonstrated that ALA exerts beneficial effects on metabolic syndrome, lipotoxic cardiomyopathy and endothelial dysfunction through the activation of AMPK in various tissues.^{78–80} Although the underlying mechanisms for AMPK regulation by ALA are poorly understood, Shen *et al.* have reported that ALA increases the intracellular calcium level in C2C12 myotubes, suggesting that CaMKK, but not LKB1, is responsible for AMPK activation.⁸¹ In the hypothalamus, where AMPK is implicated in the regulation of appetite, ALA suppresses AMPK activity, leading to reduced food intake.⁸² Further examination is required to understand the molecular mechanism of the regulation of AMPK by ALA.

Other AMPK modulators

Although intracellular energy levels are a major determinant of AMPK activity, AMPK is highly sensitive to the cellular level of reactive oxygen species (ROS).⁸³ In many cases, oxidative stress results in intracellular ATP depletion. However, recent studies have revealed that ROS can stimulate AMPK activity even without a decrease in cellular ATP.^{84,85} Oxidative modification of the AMPK α subunit appears to be a major mechanism by which AMPK is activated under conditions of oxidative stress.⁸⁶ Therefore, any modulators capable of inducing intracellular ROS generation can activate AMPK without an associated decrease in ATP levels. Such a modulator is cryptotanshinone from *Salvia miltiorrhiza Bunge*, which exerts antidiabetic⁸⁷ and anticancer effects⁸⁸ through ROS-dependent AMPK activation. DNA-damaging agents, such as cisplatin⁸⁹ or metals, including arsenite, vanadate and cobalt,⁹⁰ activate AMPK through ROS generation.

DIRECT AMPK ACTIVATORS

Several AMPK activators directly bind to and activate AMPK without any significant change in cellular ATP, ADP or AMP levels. Instead, these activators induce conformation changes in

the AMPK complex, leading to activation, possibly through a direct interaction with a specific subunit of AMPK (Table 2). The identification of A-769662 by Abbott Laboratories in 2006 provided a novel insight into the development of direct AMPK activators by demonstrating that AMPK activation with non-nucleotide ligands is possible. In addition, it opened up the possibility of developing an activator with AMPK heterotrimer specificity. Since then, numerous studies reporting direct AMPK activators have provided meaningful advances regarding isoform-specific modulators.

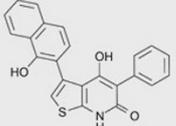
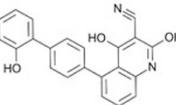
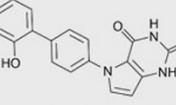
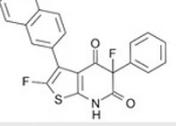
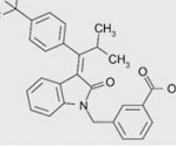
5-Aminoimidazole-4-carboxamide riboside

The first direct AMPK activator, 5-aminoimidazole-4-carboxamide riboside (AICAR), is an adenosine analog taken up into cells by adenosine transporters and phosphorylated by adenosine kinase, thus generating the AMP-mimetic, AICAR monophosphate (ZMP).^{91,92} Similarly to cellular AMP, ZMP binds to site 3 on the AMPK γ subunit. ZMP does not change the ADP:ATP ratio or alter oxygen uptake, which occurs with many AMPK activators through the inhibition of mitochondrial function.¹¹ Although ZMP is a much less potent AMPK activator than AMP in cell-free systems, AICAR directly activates AMPK in most cells because ZMP can accumulate to millimolar concentrations in cells. ZMP is a natural intermediate in the purine nucleotide synthetic pathway and is metabolized by AICAR transformylase, which catalyzes synthesis of the purine nucleotide inosinate.⁹³ Therefore, the effect of AICAR seems to be more apparent in quiescent, primary cells than in rapidly proliferating cells. Consistently with this notion, anticancer agents that inhibit AICAR transformylase, such as methotrexate and Pemetrexed, sensitize tumor cells to the AMPK-activating and growth-inhibitory effects of AICAR.^{94,95} These results indicate that AMPK participates in the chemotherapeutic effects of antifolate drugs to treat cancers. However, it should be noted that, as an AMP analog, AICAR is able to activate many other AMP-dependent enzymes, such as fructose-1,6-bisphosphatase.^{96,97}

Thienopyridone (A-769662) and benzimidazole (Compound 911) derivatives

Abbott Laboratories has developed a thienopyridone compound, A-769662, which causes allosteric activation of purified AMPK in cell-free assays.⁹⁸ This compound shows many of the metabolic effects that would be expected with AMPK activation *in vivo* (increase in fat oxidation in normal rats; decreases in body weight, plasma glucose/triglycerides and liver triglycerides in obese mice). Unlike AICAR, A-769662 shows high specificity toward AMPK. A-769662, similar to AMP, allosterically activates the AMPK complex and inhibits dephosphorylation of Thr-172 in the AMPK α subunit.^{99,100} However, A-769662 appears to use a different molecular mechanism to activate AMPK.¹⁰¹ Notably, it allosterically activates AMPK without Thr172 phosphorylation on the AMPK α subunit, which is absolutely required for AMP-dependent AMPK activation. Importantly, it requires phosphorylation of Ser108 on the AMPK β 1 subunit. Moreover, the strong synergic AMPK

Table 3 Direct AMPK activators from patent literatures

Patent number	A representative example	In vitro activity	First applicant
WO 2009124636		Activation of recombinant AMPK $\alpha 1\beta 1\gamma 2$ at 30 μM : 625%	Merck Patent GMBH
WO 2009100130		Activation of partially purified AMPK: ED_{50} < 10 μM	Mercury Therapeutics Inc.
WO 2011029855 WO 2011138307		Activation of human recombinant AMPK ($\alpha 1\beta 1\gamma$ and $\alpha 2\beta 1\gamma 2$) pEC200 (-Log(compound : concentration leading to a 2-fold AMPK activity): 5.9	GlaxoSmithKline LLC.
WO 2011080277		AMPK Activity: Ratio between the % of control (basal activity) of compound of formula at 30 μM and the % of control (basal activity) of AMP (natural substrate) at 200 μM	Poxel
WO 2011032320 WO 2011033099		Activation of human recombinant AMPK ($\alpha 1\beta 1\gamma$ and $\alpha 2\beta 1\gamma 2$) EC_{50} : 0.66 μM	Hoffmann-La Roche

activation by AMP and A-769662 has been observed both *in vitro* and *in vivo*, clearly demonstrating that A-769662 and AMP have different binding sites on the AMPK complex and different mechanisms of activation.¹⁰² Another direct AMPK activator, compound 911, has recently been identified. 911 has been reported to be 5–10-fold more potent than A-769662 in allosterically activating AMPK and preventing dephosphorylation.¹² Similarly to A-769662, 991 does not activate AMPK complexes containing the Ser108 mutation of the AMPK β subunit, suggesting that these two AMPK modulators share a similar molecular mechanism of AMPK activation. Xiao B *et al.*¹² have solved the crystal structure of the full-length human AMPK complex in the presence of A-769662 or 991. In this structure, both A-769662 and 911 are located at a site between the KD of the AMPK α subunit and the carbohydrate-binding module (CBM) of the β -subunit, a site distinct from the adenine nucleotide-binding sites on the AMPK γ subunit. Interestingly, both chemicals exhibit specificity toward AMPK complexes containing the $\beta 1$ rather than the $\beta 2$ isoform.

Salicylate (pro-drug of Aspirin)

Salicylate is a natural compound traditionally extracted from willow bark. Acetyl salicylate (aspirin) is a derivative that is easier than salicylate to take orally and is rapidly broken down to salicylate upon entering the circulation. Although cyclooxygenases (COX1 and COX2) are the established targets for aspirin, it has been reported recently that salicylate (although not aspirin) is a direct activator of AMPK.¹⁰³ In line with its

structural similarity to A-769662, salicylate appears to bind at a site that overlaps with the site targeted by A-769662. Both compounds cause allosteric activation, with salicylate antagonizing the effect of A-769662. In addition, the effects of both compounds are highly dependent on the AMPK $\beta 1$ subunit but not on AMPK $\beta 2$. Neither compound activates AMPK complexes with the Ser108 mutation of the AMPK $\beta 1$ subunit. Considering that thienopyridone (A-769662), benzimidazole (Compound 911) and salicylate derivatives activate AMPK by mechanisms different from most AMP-mimetics or ATP-depleting AMPK activators, the combination of these molecules with the indirect AMPK activators is expected to augment the effect of AMPK on pathophysiological conditions, such as metabolic disorders and cancers.^{104–107}

Compound-13

Recent screening of a chemical library containing 1,200 AMP mimetics has identified 5-(5-hydroxyl-isoxazol-3-yl)-furan-2-phosphonic acid, termed Compound-2 (C-2), and its pro-drug C-13, as potent allosteric activators of AMPK.¹⁰⁸ A subsequent study has demonstrated the molecular mechanism by which C-2 mimics the effects of AMP to stimulate AMPK.¹⁰⁹ One concern, as observed with AICAR, is the possibility that C-2 may affect AMP-regulated enzymes other than AMPK (PFK1, FBP1 and glycogen phosphorylase). However, C-2 does not affect any of these enzymes or several enzymes that use AMP as a substrate. *In vitro* cell-free assays using several AMPK complexes have revealed that C-2 is a potent allosteric activator of AMPK (EC_{50} of 10–30 nM). In fact, C-2 has been reported

to be >20-fold more potent than A769662 and more than two orders of magnitude more potent than AMP.^{98,110} In addition, C-2 and C-13 do not induce any significant change in adenine nucleotide levels. Although the precise C-2-binding sites have not been identified, evidence presented by Hunter *et al.*¹⁰⁹ has suggested that C-2 competes with AMP for binding on the AMPK γ subunit. Surprisingly, the AMPK activators C-2 and C-13 exhibit isoform specificity toward the AMPK α 1 subunit. Structural analyses of AMPK complexes^{12,13} indicate that different sequences of AMPK α 1 and α 2 subunits in the α -regulatory subunit-interacting motif-2 (α -RIM2) region, which is used to generate AMPK α isoform-specific antibodies, result in unique interactions of C-2 with one face of AMP bound at Site 3 of the γ -subunit, accounting for the selectivity of C-2 toward AMPK α isoforms. Identification of C-2/C-13 represents an example of the development of a direct and isoform-specific AMPK modulator that is distinct from A-769662 that shows a CBM-dependent AMPK β subunit specificity.¹⁰⁹

PT-1

Another small molecule activator of AMPK, PT-1, was initially isolated via a screen of compounds that activated the truncated AMPK α 1 construct containing only the KD and the AID.¹¹¹ PT-1 activates the complete AMPK α 1 β 1 γ 1 as well as the AMPK α 1 KD-AID construct but not the AMPK α 1 KD construct, suggesting that PT-1 directly binds to the cleft between the KD and the AID, thereby relieving autoinhibition. Consistently with results from a cell-free kinase assay, PT-1 has been shown to increase the phosphorylation of ACC at Ser79, a well-characterized substrate of AMPK, in L6 myotubes without any significant change in cellular AMP:ATP ratio. However, this result has been questioned by a recent report by Jensen *et al.*¹¹² showing that PT-1 indirectly activates AMPK via inhibition of the mitochondrial respiratory chain complex, thereby increasing cellular AMP:ATP and/or ADP:ATP ratios, instead of binding directly to the AMPK α 1 subunit, as previously suggested.¹¹¹ In line with the notion that PT-1 increases intracellular AMP levels, PT-1 does not activate AMPK in HEK293 cells expressing an AMP-insensitive AMPK γ 1 R299G mutant, suggesting that PT-1 functions as an indirect activator. Furthermore, this study has shown that PT-1 selectively activates the AMPK complex containing the γ 1-subunit but not γ 3 in incubated mouse muscle. The authors have proposed that the failure of PT-1 to activate γ 3-containing complexes in muscle is not an intrinsic feature of such complexes but occurs because PT-1 does not increase cellular AMP:ATP ratios in the distinct subcellular compartments containing γ 3-complexes. Therefore, the molecular details of PT-1 action should be further studied to address the questions raised by these contradictory results.

MT 63–78 (Debio0930)

Another AMPK direct modulator, MT 63–78 (Debio0930), has recently been identified to allosterically activate AMPK.¹¹³ Biochemical analysis has shown that the effect of MT 63–78

is highly selective for the AMPK complex containing the AMPK β 1 subunit, as was seen for A-769662 and salicylate. Notably, MT 63–78 strongly suppresses the growth of prostate cancer cell lines with a concomitant activation of AMPK but without any significant change in cellular ATP, ADP and AMP levels. Importantly, the growth-inhibitory effects of MT 63–78 on prostate cancers are at least 10–40 times higher than those of A-769662. In many prostate cancer models, androgen is believed to drive tumorigenesis and progression of the cancers.¹¹⁴ Therefore, androgen deprivation therapy is a first option to treat this cancer. However, in many cases, the androgen-signaling cascade is re-activated after chemotherapeutic treatments that target the androgen receptor, for example, the androgen receptor antagonist MDV3100.¹¹⁵ Upregulation of *de novo* lipogenesis by androgen in prostate cancer is also closely related to cancer development.^{116,117} Considering that AMPK negatively regulates *de novo* lipogenesis,^{92,108,118,119} the combination treatment of AMPK activators and androgen receptor inhibitors may function cooperatively as antiprostate cancer drugs. The clinical potential of this concept has been shown in a therapeutic trial. This trial showed that the suppression of *de novo* lipogenesis is the key mechanism of AMPK inhibition of growth and that MT 63–78 enhances the inhibitory effect of androgen receptor antagonist (MDV3100) on the growth of prostate cancer cells. In addition, the inhibitory effect of MT 63–78 on growth is not limited to prostate cancer cells and has also been observed in LKB1-null A549 cells and in B-RAF-mutated (V600E) KTC-1 cells. These results suggest that MT 63–78 slows the growth of a wide spectrum of cancers, thus increasing the chemotherapeutic effects of current anticancer drugs.

PERSPECTIVE

Most of the current agents that have been shown to activate AMPK in physiological trials, such as metformin, TZDs and 2-deoxyglucose, are indirect activators that inhibit oxidative phosphorylation and glycolysis, thereby increasing the ADP (AMP):ATP ratio. However, it is not always clear whether the effects of these agents are mediated by AMPK. In this sense, much effort has been focused on demonstrating the molecular mechanisms of AMPK activators and on validating the resulting physiologies on many human diseases.^{2,120} Another concern when developing AMPK activators is that there are functional differences between isoform-specific AMPK complexes. For instance, the AMPK α 2 β 2 γ 3 complex is predominantly activated by exercise in skeletal muscle,⁵ and therefore specific targeting of the AMPK α 2 β 2 γ 3. Recent studies reporting direct AMPK activators have provided meaningful advances in developing isoform-specific modulators. For the AMPK α subunit, C-2 (or a pro-drug C-13) has a preference for AMPK complexes containing the AMPK α 1 subunit.¹⁰⁸ Similarly to A-769662,⁹⁸ several compounds including 911,¹² salicylate (a pro-drug of aspirin)¹⁰³ and MT 63–78¹¹³ specifically activate AMPK β 1-containing complexes but not those containing AMPK β 2. In the case of the AMPK γ subunit, although further studies at the cellular level are required,

in vitro biochemical data have shown that PT-1 has a specificity toward AMPK complexes harboring the AMPK γ 1 subunit.¹¹¹ In addition to these activators, a number of pharmaceutical companies have filed patent applications for novel AMPK activators, which are structurally unrelated to AMP. Some representative compounds from each pharmaceutical company are listed in Table 3. Comprehensive lists of AMPK activators in the patent literature are available elsewhere.^{121,122} It is highly intriguing that, although they have been claimed to be novel, the majority of the direct AMPK activators listed in Table 3 show a close resemblance to the original thienopyridone core structure of A-769662, except for the alkene oxindole derivative reported from F. Hoffmann-La Roche AG. Given the recent reports suggesting the AMPK-independent effects of A-769662,^{100,123} further studies are needed to clarify the molecular basis of the accumulating number of direct AMPK activators, by comparing their activation mechanisms and by analyzing their profiles of selectivity across AMPK complex combinations.

One interesting aspect of AMPK activators revealed by preclinical studies is the enhanced therapeutic effects of the combination of different AMPK activators. As a master regulator of lipogenic pathway,²⁵ AMPK may be an additional chemotherapeutic target because the upregulation of fatty-acid synthesis is a hallmark of many cancers.¹²⁴ Evidence has shown that the combination of aspirin (salicylate) and Metformin effectively decreases clonogenic survival of prostate and lung cancer cells.¹⁰⁴ Consistently with this finding, the addition of fatty acids and/or cholesterol into the culture medium reverses the suppressive effects of salicylate and metformin on cell survival, indicating that the inhibition of *de novo* lipogenesis is important.^{105,106} Similarly, direct AMPK activators may open new therapeutic avenues for anticancer reagents. In the case of the conventional indirect AMPK activators, the mechanism of action requires the upstream kinase LKB1 for physiological AMPK activation. Therefore, the potential of indirect AMPK activators as anticancer drugs is limited to LKB1-deficient tumors, especially for non-small cell lung cancers, of which more than 30% have LKB1-inactivating mutations. In this aspect, direct AMPK activators may overcome this limitation. The evidence shows that the growth-inhibitory response to the AMPK activator, MT 63–78, is not affected by the status of the upstream AMPK-activating kinase LKB1.

In conclusion, the recent advances identifying direct AMPK activators make AMPK a ‘druggable’ target for many human diseases, although further studies are required to gain insight into the molecular mechanisms by which AMPK regulates its distinct and diverse downstream targets to produce physiological outcomes.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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