

# Protein kinase inhibitors block the stimulation of the AMP-activated protein kinase by 5-amino-4-imidazolecarboxamide riboside

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**Abstract** The AMP-activated protein kinase (AMPK) is the central component of a protein kinase cascade that plays a major role in energy sensing. AMPK is activated pharmacologically by 5-amino-4-imidazolecarboxamide (AICA) riboside monophosphate (ZMP), which mimics the effects of AMP on the AMPK cascade. Here we show that uptake of AICA riboside into cells, mediated by the adenosine transport system, is blocked by a number of protein kinase inhibitors. Under these conditions, ZMP does not accumulate to sufficient levels to stimulate AMPK. Our results demonstrate that careful interpretation is required when using AICA riboside in conjunction with protein kinase inhibitors to investigate the physiological role of AMPK.

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**Key words:** AMP-activated protein kinase; Metabolic regulation; Nucleoside uptake; Protein kinase inhibitor

## 1. Introduction

The AMP-activated protein kinase (AMPK) plays a major role in the regulation of energy metabolism and is activated in response to a rise in the ratio of AMP:ATP, following ATP depletion [1,2]. AMP increases AMPK activity by direct allosteric activation and also by promoting the phosphorylation of AMPK by an upstream kinase, AMPK kinase (AMPKK). Recently, several studies have demonstrated that AMPK is activated by a second mechanism that does not appear to involve changes in adenine nucleotides, although the molecular basis for this activation is not understood [3–5]. Once activated, AMPK plays a diverse role within the cell, switching-off energy utilising pathways and switching-on energy generating pathways [2,6]. All of the known functions of AMPK appear to be aimed at maintaining the energy balance within the cell, leading to the hypothesis that AMPK acts as a metabolic master switch [7]. For instance, AMPK has been implicated in the increased uptake of glucose [8] and increased fatty acid oxidation [9] in skeletal muscle following exercise. In muscle, activation of AMPK by leptin leads to increased fatty

acid oxidation [5]. The combined actions of AMPK make it a promising target for therapeutic agents aimed at alleviating the symptoms of metabolic disease states, such as type 2 diabetes and obesity. Recently, mutations in the  $\gamma 2$  isoform of AMPK have been identified in individuals suffering from hypertrophic cardiomyopathy associated with Wolff–Parkinson–White syndrome, indicating that the kinase is also important in the normal development and functioning of the heart [10,11]. Given the potential role of AMPK in human disease, considerable effort is being invested by many groups to investigate further the regulation and function of the kinase.

A major development in AMPK research was the finding that 5-amino-4-imidazolecarboxamide (AICA) riboside could be used to activate AMPK pharmacologically in cells [12,13]. AICA riboside is converted in cells to the monophosphate derivative, ZMP, which can accumulate to high levels, and mimics the effects of AMP on the AMPK cascade. Until recently, AICA riboside was the only pharmacological activator of AMPK to be described and many studies investigating the physiological consequences of AMPK activation relied solely on its use. Over the last few years several small, cell-permeable inhibitors of protein kinases have been developed that exhibit relatively high specificity [14]. Zhou et al. reported recently the identification of an inhibitor of AMPK [3]. At present this inhibitor, which they termed compound C, has not been extensively characterised, but its discovery raises the possibility of a more selective approach for modulating AMPK activity in cells. In this study we examined the use of AICA riboside in conjunction with compound C to modulate AMPK activity in muscle cells. We report a serious complication that arises with pharmacological manipulation of AMPK that should be considered whenever using this approach to investigate the physiological role of the kinase.

## 2. Materials and methods

### 2.1. Cell culture

H-2K<sup>b</sup> skeletal muscle cells [15] were grown as described previously [16]. Cells were allowed to differentiate for 4 days prior to treatment. On the day of harvesting, cells were transferred to 20 mM HEPES, pH 7.4, 140 mM NaCl, 5 mM KCl, 2.5 mM MgSO<sub>4</sub>, 1 mM CaCl<sub>2</sub>, 5 mM glucose and incubated for 30 min in the presence or absence of either 20  $\mu$ M compound C (a generous gift from Dr. G. Zhou, Merck Pharmaceuticals), 10  $\mu$ M SB202190, or 10  $\mu$ M 203580 (Calbiochem, San Diego, CA, USA). Inhibitors were dissolved in dimethyl sulphoxide and diluted into the cell media to give the appropriate concentration with a final concentration of 0.2% dimethyl sulphoxide. Cells were then incubated for a further 30 min in the presence or absence of either 500  $\mu$ M AICA riboside, 0.6 M sorbitol or 0.5 mM dinitrophenol (DNP), in the continued presence of inhibitor where added.

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**Abbreviations:** AICA, 5-amino-4-imidazolecarboxamide; AMPK, AMP-activated protein kinase; MAPK, mitogen-activated protein kinase

## 2.2. AMPK activity measurements

Following treatment, cells were lysed in a minimal volume of ice-cold 50 mM Tris-HCl, pH 7.5, 50 mM NaF, 5 mM Na pyrophosphate, 1 mM EDTA, 1 mM dithiothreitol, 10% (v/v) glycerol, 1 mM benzamide, 0.1 mM phenylmethane sulphonylfluoride, 1% (v/v) Triton X-100 (buffer A). Cell debris was removed by centrifugation at  $13000\times g$  for 5 min. AMPK was immunoprecipitated from the lysates (100  $\mu$ g protein) with an anti- $\beta$  antibody bound to protein-A Sepharose [17]. Immune complexes were washed with  $3\times 1$  ml buffer A and AMPK activity determined using the SAMS peptide assay [18].

For *in vitro* assays AMPK was partially purified from rat liver, up to the diethylaminoethyl (DEAE)-Sepharose step [19]. AMPK was diluted in buffer A and assayed using the SAMS peptide in the presence or absence of varying concentrations of inhibitor. Several dilutions of AMPK were used to ensure the assays were carried out within a linear range.

## 2.3. Nucleotide measurements

Nucleotides present in perchloric acid extracts of H-2K<sup>b</sup> cells were analysed by ion-exchange chromatography, as described previously [4].

## 2.4. Nucleoside uptake

Adenosine uptake into H-2K<sup>b</sup> cells was determined by measuring the disappearance of [<sup>3</sup>H]-adenosine (Amersham Biosciences) from the media [20]. Radiolabelled adenosine (2  $\mu$ Ci/ml) was added to the cells that had been incubated in the presence or absence of inhibitor for 30 min. Aliquots (40  $\mu$ l) of the media were removed after 0, 2, 5, 15 and 30 min and radioactivity remaining in the media determined by scintillation counting.

## 3. Results

We have previously reported the activation of AMPK in H-2K<sup>b</sup> cells by a number of different treatments, including AICA riboside, hyperosmotic stress (0.6 M sorbitol) and DNP [4,16]. In order to characterise further the physiological role of AMPK we decided to make use of an inhibitor of the kinase that has recently been identified by Zhou and colleagues [3]. We were also interested in determining whether the mechanism of activation of AMPK by hyperosmotic stress involved the p38 mitogen-activated protein kinase (MAPK). This pathway is activated in response to hyperosmotic stress [21] and has been implicated in the AMPK-mediated stimulation of glucose transport [22]. We therefore determined the effect of compound C (20  $\mu$ M final concentration) and two inhibitors of p38 MAPK, SB202190 (10  $\mu$ M final) and SB203580 (10  $\mu$ M final), on the activation of AMPK in H-2K<sup>b</sup> cells in response to either AICA riboside, hyperosmotic stress or DNP. As can be seen from the results in Fig. 1, all three kinase inhibitors completely blocked the activation of AMPK by AICA riboside, but had no significant effect on the activation in response to hyperosmotic stress or DNP. Previous results have demonstrated that compound C inhibits AMPK *in vitro* [3], whereas SB202190 and SB203580 have no effect on AMPK activity [14]. In the present study we confirmed these previous findings (data not shown).

Since our results indicated that the inhibitors only blocked the activation of AMPK by AICA riboside we examined their effect on the level of ZMP accumulation in H-2K<sup>b</sup> cells. Fig. 2 shows the nucleotide profiles from cells treated with AICA riboside in the presence or absence of the inhibitors. All three inhibitors prevent the accumulation of ZMP that is seen in control cells incubated with AICA riboside. AICA riboside is thought to be transported into cells via the adenosine transport system [13]. Therefore, we examined the effect of the inhibitors on adenosine uptake in H-2K<sup>b</sup> cells (Fig. 3). All

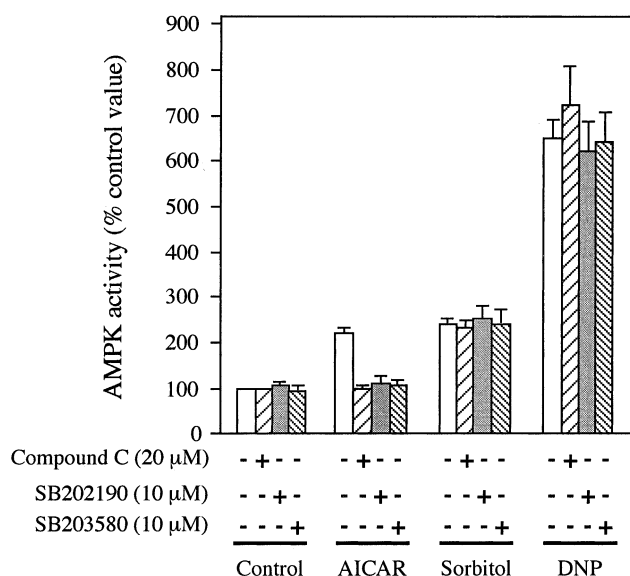


Fig. 1. Effect of protein kinase inhibitors on AMPK activity in H-2K<sup>b</sup> cells. Cells were incubated in the presence or absence of compound C (20  $\mu$ M), SB202190 (10  $\mu$ M) or SB203580 (10  $\mu$ M) for 30 min prior to the addition of either 500  $\mu$ M AICA riboside (AICAR), 0.6 M sorbitol or 0.5 mM dinitrophenol (DNP). After a further 30 min, cells were lysed and AMPK activity in anti- $\beta$  immunoprecipitates measured using the SAMS peptide assay. Results shown are the mean ( $\pm$  S.E.M) of 3–11 independent measurements and are plotted as the percentage of activity in control cells incubated in the absence of inhibitor or activator.

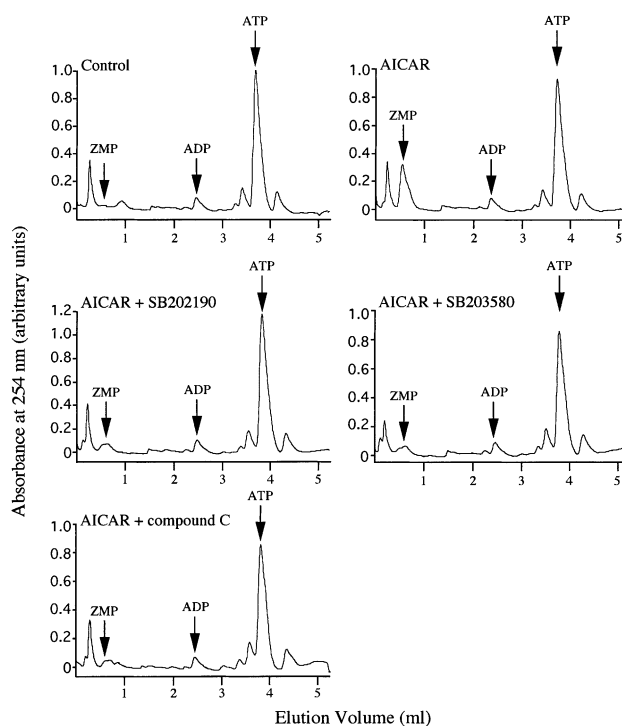


Fig. 2. Effect of inhibitors on ZMP accumulation in H-2K<sup>b</sup> cells. Cells were incubated in the presence or absence of either compound C (20  $\mu$ M), SB202190 (10  $\mu$ M) or SB203580 (10  $\mu$ M) for 30 min before the addition of 500  $\mu$ M AICA riboside. After a further 30 min incubation, cells were lysed by the addition of perchloric acid and nucleotides analysed by ion-exchange chromatography. The elution of ZMP, indicated by an arrow, was identified by co-migration with a standard. The results shown are representative of two to four independent experiments.

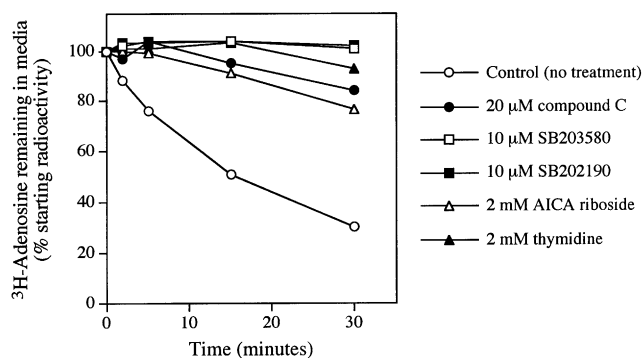


Fig. 3. Effect of inhibitors on adenosine uptake. The uptake of adenosine into H-2K<sup>b</sup> cells was determined by measuring the rate of disappearance of [<sup>3</sup>H]adenosine from the cell culture medium in the presence or absence of various compounds. The results shown are the mean values of three to four independent experiments which varied by less than 10% and are plotted as the percentage of radioactivity remaining relative to the starting amount (time zero).

three inhibitors significantly reduced the uptake of radiolabelled adenosine from the media. As expected, addition of thymidine (2 mM) competed for uptake of adenosine. Importantly, AICA riboside (2 mM) also blocked adenosine uptake, indicating that uptake of this nucleoside is mediated by the adenosine transport system.

#### 4. Discussion

During the course of our studies on the physiological role of AMPK we wanted to make use of protein kinase inhibitors in order to characterise the events upstream and downstream of AMPK activation. Our initial interest focussed on inhibition of AMPK itself and inhibition of the p38 MAPK pathway, which has recently been implicated in the AMPK-mediated activation of glucose transport [22]. Compound C was recently identified as an inhibitor of AMPK [3], although to our knowledge its specificity has not been tested extensively. In contrast, SB202190 and SB203580 are two widely used inhibitors of p38 MAPK [14]. All three inhibitors blocked the activation of AMPK by AICA riboside, but had no significant effect on AMPK activity in response to hyperosmotic stress or the mitochondrial uncoupling agent, DNP. These results are somewhat paradoxical since compound C might be expected to inhibit AMPK activity under all conditions whereas SB202190 and SB203580 have no effect on AMPK activity *in vitro* (this study and [14]). However, the finding that all three inhibitors prevent the accumulation of ZMP following incubation with AICA riboside offers a plausible explanation for our results. The failure to accumulate ZMP appears to be due to a reduction in uptake of AICA riboside, as all three inhibitors significantly reduced the uptake of adenosine into H-2K<sup>b</sup> cells. We have not studied the mechanism by which the inhibitors reduce nucleoside uptake, although it seems unlikely that it is due to inhibition of AMPK or p38 MAPK, since SB202190 and SB203580 have no direct effect on AMPK activity and compound C has no effect on p38 MAPK  $\alpha$  or  $\beta$  activity *in vitro* (A. Woods and D. Carling, unpublished data). Like most protein kinase inhibitors, the three compounds we studied act by competing for ATP and it is possible that the adenosine transport system is sensitive to this type of mechanism. Whilst we have not determined

whether the inhibition of uptake is due to an effect on transport, or phosphorylation of AICA riboside catalysed by adenosine kinase, what is clear from our results is that ZMP accumulation is sensitive to protein kinase inhibitors, and this should be given appropriate consideration wherever applicable. During the review process of our paper a report from Huang et al. described the inhibition of nucleoside transport by p38 MAPK inhibitors [23]. Their results suggest that the inhibition of uptake of AICA riboside that we observe is probably due to a direct effect of the protein kinase inhibitors on nucleoside transport.

Our results also indicate that the effects of compound C are lost during isolation of AMPK by immunoprecipitation. This is not surprising since the immune complexes are washed extensively before measuring AMPK activity. These results do not preclude the possibility that AMPK activity *in situ* is inhibited. Indeed, that this is the case seems likely based on the results of AMPK activation by metformin in hepatocytes [3]. In this study, compound C was shown to prevent the inactivation of acetyl-CoA carboxylase following incubation with either AICA riboside or metformin. Interestingly, however, compound C was much less effective in preventing inactivation of acetyl-CoA carboxylase following incubation with metformin, suggesting that some of the effect observed following treatment with AICA riboside may have been due to a reduction in ZMP accumulation. In another study, SB203580 was used to implicate p38 MAPK in the AMPK-mediated increase in glucose transport following activation with AICA riboside [22]. In this case alternative approaches were included to demonstrate a role for p38 MAPK in this response, although the levels of ZMP were not determined.

Over the last few years interest in the AMPK cascade has grown considerably, to the extent that there is now more than one paper published on AMPK per week. Many studies investigating the physiological role of AMPK have relied on the use of AICA riboside to activate the kinase in animal or cell-based model systems. As is the case with any study, care must be taken when interpreting the results obtained from the use of a pharmacological agent. This point has been made recently for the use of protein kinase inhibitors in the study of physiological processes [14]. The results of our current study reveal an important limitation when using AICA riboside in conjunction with protein kinase inhibitors to modulate AMPK activity. Based on our findings we would urge investigators working on AMPK to analyse all their data obtained with the use of AICA riboside extremely rigorously. Specifically, we recommend that the level of ZMP in tissues or cells incubated with AICA riboside is determined to allow direct correlation with AMPK activity measurements. It is clear from the literature that this is often not the case. Secondly, we suggest that alternative approaches should be used to activate AMPK. The recent finding that metformin activates AMPK [3], by a mechanism that appears to be independent of changes in adenine nucleotides [4], coupled with the development of molecular tools for modulating AMPK activity [24], makes this a realistic possibility. Implementation of these basic recommendations will minimise erroneous conclusions from being made.

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