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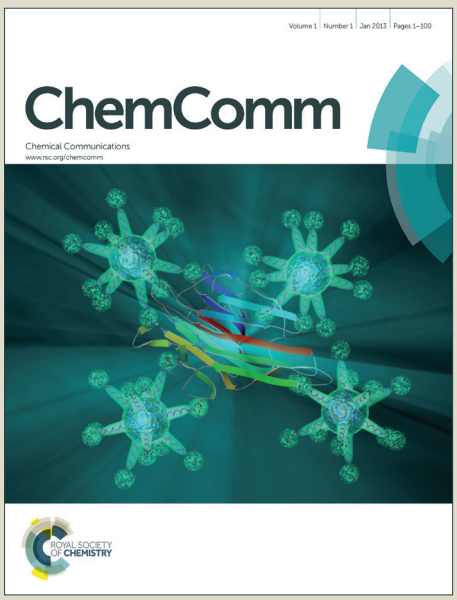

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Benzothiazole Derivatives Augment Glucose Uptake in Skeletal Muscle Cells and Stimulate Insulin Secretion from Pancreatic β-Cells via AMPK Activation

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Adenosine Monophosphate-Activated Protein Kinase (AMPK) has been identified as one of the major targets for antidiabetic drugs. This study describes two AMPK-activating agents 2-(benzo[d]thiazol-2-ylmethylthio)-6-ethoxybenzo[d]thiazole and 2-(propylthio)benzo[d]thiazol-6-ol, that increase the rate of glucose uptake in L6 myotubes and also augment glucose-stimulated insulin secretion in INS-1E β-cells and rat islets. We believe that such unique bi-functional compounds can be further used for the development of a new class of antidiabetic drugs.

Type two Diabetes Mellitus (T2DM) is characterized by insulin resistance, decreased rate of glucose disposal, and a declining ability of β-cells to produce and secrete insulin.[1] Often, severe hypoinsulinemia characterizes the advanced stages of T2DM.[2] The combination of insulin deficiency and insulin resistance in the pathogenesis of T2DM would necessitate bi-functional compounds that increase glucose utilization in peripheral tissues and restore the β-cell insulin secretory capacity in order to obtain optimal therapeutic effect.[3] Activation of AMPK (an universal regulator of body energy homeostasis) might be potentially used for achievement of such bi-functional effect in diabetic patients.

AMPK stimulation in myotubes or intact skeletal muscles leads to glucose transporter 4 (GLUT-4) translocation to the plasma membrane and consequently an augmented rate of glucose uptake.[4] In contrast with this widely accepted mechanism of AMPK dependent regulation of the rate of glucose uptake in skeletal muscles, there are some serious incongruities in the literature about the precise role of AMPK in insulin secretion regulation in β-cells in vitro and in vivo. For updated review see Fu A., et al.[5] The picture become even more complex when considering the fact that some effects of pharmacological agents used to activate AMPK in β-cells are not derived from the direct activation of AMPK.[6] On the other hand, genetic disruption of AMPK in β-cells also led to complex effects, causing impaired insulin release in vivo but stimulated secretion in vitro.[7,8] In addition, the isoform composition of AMPK in different immortal lines, in isolated islets and in intact animals also can be diverse. The enzyme is a heterotrimer complex composed of three subunits: a catalytic α, and two regulatory β and γ subunits, which are encoded by 7 different genes: α1, α2, β1, β2, γ1, γ2, and γ3.[9] The tentative possible combination of these genes creates 12 isoenzymes which are differently distributed in the tissues of different species.[10] Moreover, discrepancy in the results could be explained also by physiological differences between β-cell in immortal lines, in isolated islets and in intact animals.[4] Finally, the different β-cell preparations and maintenance from study to study may also explain the disparate results.[4,11]

Taking together all the parts of this complex picture, it is not surprising that some contradicting results are reported. Briefly, it was shown that activation of AMPK lead to decreasing of insulin secretion and even to β-cell apoptosis.[12,13] For example, Da Silva et al. showed that in rat islets and in MIN6 β-cells, AMPK affected the upstream regulating factors of insulin release and block glucose-stimulated insulin secretion.[14] On the other hand, inactivation of AMPK led to the unregulated release of insulin. Alternatively, many other research groups claimed that indeed AMPK activation increased insulin secretion.[15,16] For instance, it was shown in the rat insulinoma cells that raised AMPK activity induced glucose like stimulating electrical behavior of the cells.[17] Based on these results, the authors proposed that K_{ATP} opening (and consequently insulin secretion) in response to lowered glucose concentration requires AMPK activity.[17] Several in vivo studies also support the positive involvement of AMPK in insulin secretion. For instance, it was shown that mice lacking both AMPKα subunits in the β-cell displayed normal body weight and increased insulin sensitivity, but were greatly insulin-deficient with significant changes in the morphology of the β-cell.[8] Moreover, AMPKβ transgenic mice were glucose-intolerant and showed malfunctioning insulin secretion. Interesting observation related to the role of the exact isofom of AMPK in regulating insulin secretion in mice was made by Beall et. al.[7] They noted that AMPKα2 activity was necessary for normal function of the β-cell.

We have recently reported that 2-((benzo[d]thiazol-2-ylmethyl)thio)-6-ethoxybenzo[d]thiazole (**1**) augmented the rate of glucose uptake in rat L6 myotubes by direct activation of AMPK.[11,18] We have tested the capacity of all compounds listed in this study,

including **1**, to modify glucose-stimulated insulin secretion (GSIS) from the INS-1E β-cell line. Furthermore, other benzothiazole derivatives were planned, synthesized and similarly screened in INS-1E cells and L6 myotubes.

Supplemental Chart 1 depicts eight compounds from our previous study that augmented glucose transport in L6 myotubes in an AMPK-dependent manner.[18] Additional 11 compounds (nine novel and two known) were synthesized based on structure similarities to **1** or its moieties, as shown in Supplemental Scheme 1. The 6-ethoxybenzo[d]thiazole-2-thiol (**3**) was used as a starting molecule for synthesis of 2-(propylthio)benzo[d]thiazol-6-ol (**9**), 2-(isopropylthio)benzo[d]thiazol-6-ol (**10**), 2-(octylthio)benzo[d]thiazol-6-ol (**11**), 2-((2-(1,3-dioxan-2-yl)ethyl)thio)benzo[d]thiazol-6-ol (**12**), 2-((6-ethoxy-1-oxidobenzo[d]thiazol-2-yl)sulfonyl)acetic acid (**13**), 3-((6-ethoxybenzo[d]thiazol-2-yl)sulfonyl)propanoic acid (**14**), and 1,3-bis((6-ethoxybenzo[d]thiazol-2-yl)thio)propan-2-one (**15**).

Briefly, the phenol moiety in **3** was obtained using aluminum chloride (III) as a reagent for the de-etherification. Principally, two functional groups (free thiol and phenol) in 2-mercaptobenzo[d]thiazol-6-ol could be converted to ethers via alkylation with the corresponding alkylhalides (in the presence of sodium hydride). However, in our hands only thioether derivatives (**9**, **10**, **11** and **12**) were formed. Using a similar alkylation reaction, the carboxylic acid moiety in two other compounds were converted to the correspondent sulfonyls (**13** and **14**) via oxidation of the sulfur atom by mCPBA in THF. In addition, **15** was obtained by conjugation of the 1,3-dichloroacetone with two molecules of **3**.

To obtain 3,3'-(ethane-1,1-diylbis(sulfanediyl))dipropionic acid (**16**), the free thiol group of 3-mercaptopropanoic acid was conjugated with acetaldehyde in the presence of BF_3-diethylether in chlorophorm. Three other compounds, 3-methylimidazo[2,1-b]thiazole (**17**), 3-methylbenzo[4,5]imidazo[2,1-b]thiazole (**18**) and 3-methyl-7-nitrobenzo[4,5]imidazo[2,1-b]thiazole (**19**), were synthesized by alkylating thiol groups of their imidazole moiety with 1,3-dichloropropan-2-one, following direct cyclization of the substituted alkyl chain to obtain the corresponding two- or tricycle aromatic derivatives.[19]

The effect of these 18 compounds on GSIS was studied in INS-1E β-cells, as described before.[20] Supplemental Table 1 shows that only the benzothiazole derivatives, **1** and **9**, exhibited significant positive effects in this assay. Even most structurally closed to **9** but more bulky compound **10** did not stimulate the GSIS. Thus, these two compounds were taken for further investigation.

Supplemental Figure 1 shows that both **1** and **9** augmented GSIS in a dose- and time-dependent manner. Half maximal and maximal effects of **1** on GSIS were obtained with 17.5 and 25 μM, respectively (Supplemental Figure 1A). Noteworthy, the stimulatory effect of **1** gradually decreased above 25 μM. The time course analysis (Supplemental Figure 1B) **1** showed at least a 5-h lag before **1** maximally augmented GSIS at 12 and 24 h of incubation. Compound **9** was more potent than **1**: half maximal and maximal effects were observed with 5 and 10 μM, respectively (Supplemental Figure 1C). Moreover, the stimulatory effect of **9** was already evident after 5 h of incubation; it reached its maximum at 12 h and remained at this level up to 48 h (Supplemental Figure 1D). The efficacy of **1** and **9** was similar, nearly two-fold increase in GSIS in cells exposed to 16.7 mM glucose. Importantly, the basal rate of insulin secretion, measured in cells exposed to 3.3 mM glucose, was unaltered in the presence of **1** or **9**. Total content of insulin was not affected by both compounds. The vehicle alone had no effect on insulin secretion (data not shown).

We have previously shown that **1** is a direct activator of AMPK in L6 myotubes[18]; therefore, its ability to activate AMPK in INS-1E cells was also investigated. Supplemental Figure 2A indicates that 17.5 μM of **1** increased the phosphorylation Thr^{172} in AMPK 2.5 fold between 2.5 - 5 h of incubation and that this effect vanished after 12 h. The transient **1**-dependent activation of AMPK, which precedes its aug-menting effect on GSIS, suggests a promoting and/or sensitizing role of AMPK in the upregulation of insulin secretion from β-cells. The pattern of AMPK activation by **9** was strikingly different (Supplemental Figure 2B). Thr^{172} phosphorylation was nearly maximal (3.5 fold) within 10 min of incubation and was maximal (3.8 fold) at 25 min. A gradual decrease in the activation of AMPK started already at 1 h of incubation, but remained significantly higher than the basal level throughout the 48-h incubation period (Supplemental Figure 2B). The AMPK activator AICAR (AIC, [(2R,3S,4R,5R)-5-(4-carbamoyl-5-aminoimidazol-1-yl)-3,4-dihydroxyoxolan-2-yl] methyl dihydrogen phosphate) that induces Thr^{172} phosphorylation in AMPK served as a positive control in this study (Supplemental Figure 2 A and B)[21].

To further investigate the role of AMPK phosphorylation on insulin secretion from INS-1E cells we used the AMPK phosphorylation inhibitor compound C.[22] Supplemental Figure 2C and D show that in addition to decreasing GSIS from untreated INS-1E cells, compound C abolished the augmenting effects of **1** and **9**. In contrast, Langelueddecke *et al.* reported that compound C stimulated insulin secretion from INS-1E cells.[23] However, the same study also claimed that AICAR (the molecule with opposite pharmacological effect on AMPK) also increased insulin secretion. The maximal effect of both compounds on the GSIS was observed in 12 h and the maximal level of induced phosphorylation of AMPK by the compounds was detected for **1** in 2.5 h and for **9** in 25 min (although the effect continues for 48 h). This non-linear correlation between AMPK activation and the insulin secretion might indicate that activation of AMPK is an early upstream event and additional mediators are involved in the signal transduction.

We have shown before that **1** increases glucose uptake in L6 myotubes in a dose- and time-dependent manner by activating AMPK.[18] Similarly, **9** augmented 3-fold the rate of glucose transport in L6 myotubes in a dose- and time-dependent manner (Supplemental Figure 3A and B): half maximal and maximal stimulatory effects were observed with 20 and 40-50 μM of **9**, respectively. The time-course analysis with 37.5 μM of **9** showed increased rate of glucose transport already within 20 min of incubation, reaching near-maximal effect at 1 h, which was sustained until 24 h. Supplemental Figure 3C shows that **9** markedly increased the abundance of GLUT-4 in the plasma membrane of L6 myotubes. This assay utilized the GLUT4myc-expressing L6 cell line, which allows for a simple colorimetric immunodetection of GLUT-4myc content in the plasma membrane.[24] The additive effect of maximal concentrations of insulin and **9** on GLUT-4myc abundance indicates that possibly each agent mobilized GLUT-4-rich vesicles form the cell interior to the plasma membrane by utilizing independent cellular mechanisms. This idea was also supported by the results presented in Supplemental Figure 4, showing additive effect of **9** and insulin on the rate of glucose uptake in L6 myotubes. Figure 3C also showed that the total cell content of GLUT-4 was not altered in the presence of **9** during 5 h of incubation. The ability of compound C to prevent **9**-induced stimulation of glucose uptake suggests a role for AMPK in this mechanism (Supplemental Figure 3D). This was further proven when the level Thr^{172}-phosphorylation in AMPK was determined in **9**-treated L6 myotubes (Supplemental Figure 3E): maximal activation of AMPK was evident already within 10-25 min of incubation. AMPK remained then fully activated throughout the 24-h incubation. The disparate time-dependent activation of AMPK

by **1** and **9** could result from their different metabolic stability. Nonetheless, our results indicate that early AMPK stimulation by **1** or **9** was sufficient to induce long-term effects (up to 24 hours) on GSIS in β-cells and glucose uptake in myotubes. Long-term activation of AMPK seems not required for maintaining these stimulatory effects over several hours.

Acetyl-CoA carboxylase (ACC) is considered an important downstream target that is physiologically regulated by AMPK in L6 myotubes.[25] Nevertheless, ACC was not identified as an AMPK downstream target in the regulation of insulin secretion. Thus, AMPK-induced phosphorylation of ACC was only determined in **9**-treated L6 myotubes. Supplemental Figure 3E shows that **9** significantly induced Ser^{79}-phosphorylation in ACC. AICAR served as positive control in this experiment. To further investigate the hypothesis that insulin and **9** utilize independent mechanisms to translocate GLUT-4 to the plasma membrane, the ability of both to activate the insulin receptor down-stream target AKT/PKB by Ser^{473} phosphorylation was studied. Supplemental Figure 3F shows the expected insulin-induced activation; however, **9** had no noticeable effect on AKT/PKB phosphorylation throughout the entire 24-h incubation period. The total cell content of AMPK, ACC and AKT/PKB remained unchanged in **9**-treated myotubes (Supplemental Figure 3E and F).

Additionally, direct activation of human recombinant AMPK (α1, β1, γ1) by compound **9** was identified (Supplemental Figure 5). The known direct activator of AMPK, PT-1, was used as a positive control. Compound **9** dose dependently increased the kinase activity of AMPK, although 9 was less active in comparison with **1** (the effect of **1** is described by Meltzer-Mats, et al.).[18] In the 20 μM, **1** caused a 4.3 fold effect compared with only a 1.6 fold effect of **9**. The maximal direct activation effect of **9** on AMPK kinas activity (around 3.0 fold) was obtained in concentration of 40 μM. These findings suggest that **9** also interacts with AMPK and enhances its catalytic activity. The difference between the concentrations of the two compounds for activation of AMPK might be explained by the less bulky structure of compound **9** compared to compound **1**, which does not allow stronger binding of **9** to AMPK.

To exclude the possibility that the sustained activation of AMPK could be detrimental to INS-1E and L6 cells, we assessed their viability following exposure to **1** and **9** using the MTT assay.[26] Supplemental Figure 6 shows that the viability of INS-1E cells and L6 myotubes exposed to increasing concretions of both agents remained intact up to 48 h of incubation. INS-1E are an immortalized cell line and may not entirely reflect the function of primary β-cells within islets of Langerhans.[27] Therefore, we also studied the effect of **1** and **9** on GSIS in freshly isolated rat islets (Figure 1) and found that both compounds significantly increased GSIS in this more closed to the physiological conditions model (2.54 and 1.56 fold, respectively). We suppose that the difference in the effect in isolated islets might be explained by significant difference in the lipophilicity of the two tested molecules. The calculated LogP of compound **1** is 4.63±0.73 and 4.34±0.14 determined experimentally by us.[18] The calculated LogP of the compound **9** is 3.22±0.71. Thus compound **9** is much more hydrophilic (due to the presence of free phenol group) and therefore its intracellular penetration rate may be lower than **1** which has in corresponding position a lipophilic ethyl group. In addition, the ability of compound **9** to activate AMPK directly is lower than compound **1**. This, may also explain the lower activity of compound **9** in rat islets.

In summary, we showed that compounds **1** and **9** increased glucose uptake in myotubes in a non-insulin-dependent manner and augmented GSIS from β-cells. These compounds stimulated AMPK in the two types of cells, while pharmacological inhibition of the enzyme abolished these effects. The bi-functionality of **1** and **9** is of importance, as they represent a new class of antidiabetic compounds that may reduce blood glucose levels by amplifying the glucose effect on insulin secretion on the one hand, and augment glucose uptake and utilization in skeletal muscle on the other. Such a unique combination of pharmacological effects on distinct key pathogenic mechanisms in diabetes was never reported before. Moreover, the finding that insulin and the compounds increase glucose uptake in myotubes in an additive manner further points to their distinctive potential role in pharmacotherapy of T2DM. It remains to be investigated whether these compounds can restore insulin secretion in failing β-cells and/or improve glucose uptake in insulin resistant skeletal muscles in diabetic animals.

Notes and references

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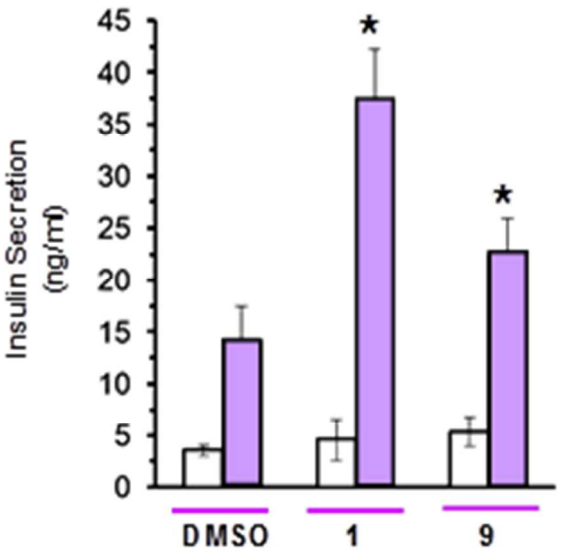

Figure 1. Effect of **1** and **9** on insulin secretion in rat islets. Isolated rat islets, maintained in complete medium, were exposed to 25 μM of **1** or **9** for 24 h. Control islets received the vehicle [0.1% (v/v) DMSO]. The islets were then washed and taken for the standard static GSIS (1-h incubation in KRB with 3.3 mM glucose (white bars), followed by 1-h with 16.7 mM glucose (violet bars), as described under "Materials and Methods" in Electronic Supplementary Information