Journal of Enzyme Inhibition and Medicinal Chemistry

ISSN: 1475-6366 (Print) 1475-6374 (Online) Journal homepage: http://www.tandfonline.com/loi/ienz20

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To cite this article: Olga Scudiero, Ersilia Nigro, Maria Ludovica Monaco, Giorgia Oliviero, Rita Polito, Nicola Borbone, Stefano D'Errico, Luciano Mayol, Aurora Daniele & Gennaro Piccialli (2015): New synthetic AICAR derivatives with enhanced AMPK and ACC activation, Journal of Enzyme Inhibition and Medicinal Chemistry, DOI: 10.3109/14756366.2015.1063622

To link to this article: http://dx.doi.org/10.3109/14756366.2015.1063622

Journal of Enzyme Inhibition and Medicinal Chemistry

http://informahealthcare.com/enz
ISSN: 1475-6366 (print), 1475-6374 (electronic)

J Enzyme Inhib Med Chem, Early Online: 1–6
 DOI: 10.3109/14756366.2015.1063622

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RESEARCH ARTICLE

New synthetic AICAR derivatives with enhanced AMPK and ACC activation

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Abstract

5-Aminoimidazole-4-carboxamide riboside (AICAR) has an important role in the regulation of the cellular metabolism showing a broad spectrum of therapeutic activities against different metabolic processes. Due to these proven AICAR properties, we have designed, synthesized and tested the biological activity of two ribose-modified AICAR derivatives, named A3 and A4, in comparison to native AICAR and its 5′-phosphorylated counterpart ZMP. Our findings have shown that A3 and A4 derivatives induce the phosphorylation of 5′-AMP activated protein kinase α (AMPKα), which leads to the inhibition of acetyl-CoA carboxylase (ACC), and down-regulate the activity of the extracellular signal-regulated kinases (ERK1/2). Cytotoxicity tests demonstrated that A3 and A4 do not significantly reduce cell viability up to 24 h. Taken together our results indicate that A3 and A4 have a comparable activity to AICAR and ZMP at 0.5 and 1 mM suggesting their potential use in future pharmacological strategies relating to metabolic diseases.

Keywords

AICAR, AMPK, ACC, cell lines cytotoxicity, p-ERK1/2

History

Received 1 April 2015
Revised 7 May 2015
Accepted 11 May 2015
Published online 6 October 2015

Introduction

During the last two decades, metabolic diseases have become the most common chronic disease group worldwide[1]. 5-Aminoimidazole-4-carboxamide riboside (AICAR) is a potent activator of 5′-AMP activated protein kinase (AMPKα), a protein kinase that increases fatty acid oxidation in multiple tissues and also stimulates the uptake and the transport of glucose as well as glycolysis[2–4]. At the same time, the activation of AMPKα also results in inhibitory effects on lipogenesis in the liver in an opposite manner compared to those of insulin. In muscle and liver, the activation of AMP leads to the inactivation of acetyl-CoA carboxylase (ACC) increasing fatty acid oxidation and inhibiting triacylglycerol synthesis. The AMPKα pathway is also implicated in the regulation of cell proliferation and its activation by AICAR could result in pro-apoptotic events[5–8]. In addition, AICAR can also exert an influence on the extracellular signal-regulated kinase (ERK1/2), a protein involved in cell proliferation whose regulation plays an important and widely investigated role in anti-cancer approaches[9]. Therefore, AICAR could be considered as an important tool in the treatment of obesity and related co-morbidities.

In this context, we have focused on AICAR due to its direct or indirect involvement with a number of important metabolic or signaling events[1–4]. Unfortunately, AICAR shows some limitations: it has a short half-life in cells; it does not cross efficiently the blood-brain barrier and it is poorly absorbed by the gastrointestinal tract. These considerations, as well as the presence of some side effects in the administration of AICAR[10], have stimulated the research of new structurally related derivatives/analogues with improved and well-targeted biological properties.

To achieve this aim, following on from previous studies[11,12], we have designed and synthesized two new ribose-modified AICAR derivatives, named A3 and A4, that can be considered more lipophilic AICAR analogues (Figure 1). We have evaluated some metabolic effects of these compounds on A549, HepG2 and C2C12 cell lines verifying the phosphorylation status of AMPKα and ACC and the regulation of the ERK1/2 enzyme. Furthermore, we have investigated the cytotoxicity effects of A3 and A4 and evaluated their stability in human serum. We anticipate here that these AICAR derivatives activate AMPKα, and ACC probably via AMPKα and down-regulate ERK1/2. Furthermore, we have demonstrated that both A3 and A4 were not toxic for cells up to 24 h.

Methods

Reagents and synthesis

Solid support **1** (Scheme 1) exploits a 4-methoxytrityl chloride resin (MMTCl, 1% divinylbenzene, 200–400 mesh, 1.3 mmol/g loading) which was purchased from CBL (Greece)[13]. All reagents

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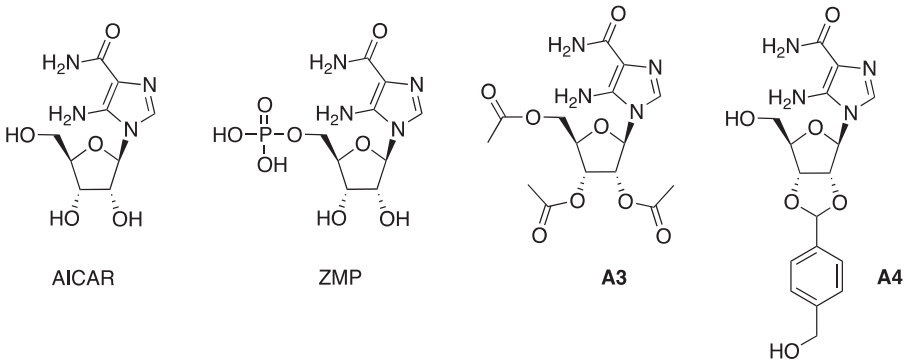
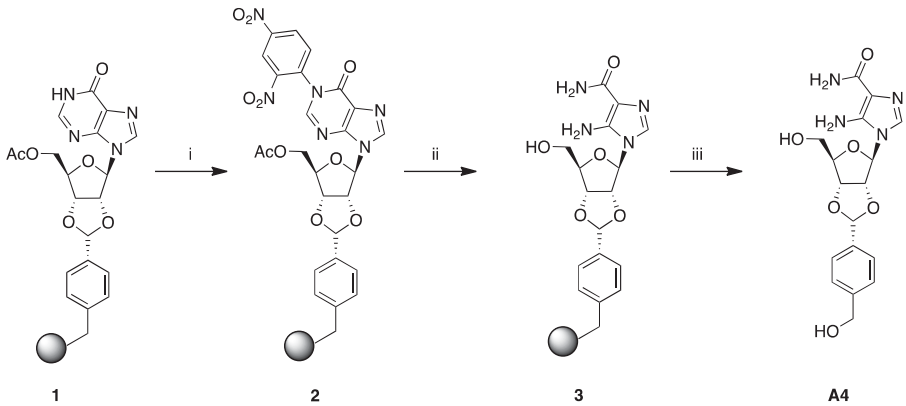


Figure 1. Structures of AICAR and ZMP and derivatives A3 and A4.



Scheme 1. Reagents and conditions: (i) DNCB, K_2CO_3, DMF, 80°C, 3 h; (ii) EDA, DMF, 50°C, 8 h; (iii) TFA (2% in dry DCM), r.t., 8 min.

were obtained from commercial sources (Sigma-Aldrich, Germany) and were used without further purification. The reactions on solid phase were performed using glass columns (10 mm diameter, 100 mm length) with fused-in sintered glass-disc PO (bore of plug 2.5 mm), which were shaken in an orbital shaker, or in round-bottomed flasks, when the reactions required high temperatures. The ^{1}H-NMR spectra were recorded on a Varian Mercury Plus 400 MHz in CD_3OD as solvent. The chemical shifts were reported in parts per million (δ) relative to the residual solvent signal (^{1}H: CD_2HOD 3.31; ^{13}C: CD_3OD 49.0). The abbreviations s, d and m stand for singlet, doublet and multiplet, respectively. The UV spectra were recorded on a Jasco V-530 UV spectrophotometer. The High Resolution MS spectra were recorded on a Bruker APEX II FT-ICR (9.4 T) mass spectrometer using electrospray ionization (ESI) in positive mode.

Synthesis of A3

5-Aminoimidazole-4-carboxamide riboside (AICAR; 100 mg, 0.39 mmol) was dissolved in a solution of acetic anhydride in pyridine (3:7, v/v, 1 mL, 15 h, r.t.). The crude, dried under reduced pressure, was purified on a silica gel column eluted with increasing amounts of CH_3OH in DCM (from 0% to 10%). The fractions eluted with 10% of CH_3OH were collected and then evaporated, affording pure A3 (142 mg, 95%) as a foam. El. An. Calcd. for $C_{15}H_{20}N_4O_8$: C, 46.88; H, 5.25; N, 14.58. Found: C, 46.92; H, 5.28; N, 14.62. ^{1}H-NMR (400 MHz, CD_3OD) δ 7.40 (s, 1H, 2-H), 5.88 (d, $J = 6.6$ Hz, 1H, 1′-H), 5.58 (m, 1H, 2′-H), 5.37 (m, 1H, 3′-H), 4.48–4.27 (complex signal, 3H, $2 \times 5'$-$H_{a,b}$ and 4′-H), 2.14 (s, 3H, CH_3), 2.10 (s, 3H, CH_3), 2.06 (s, 3H, CH_3). ^{13}C-NMR (100 MHz, CD_3OD) δ 170.6, 170.4, 170.1, 167.5, 136.8, 133.3, 115.7, 88.0, 80.9, 73.1, 72.6, 62.1, 21.3, 21.0, 20.8; UV (MeOH) λ_{max} 267 nm; *m/z* 407.1184 (HRESIMS) ($[M + Na]^+$, requires 407.1179).

Synthesis of A4

To the solid support **1** (1.0 g, 0.87 mmol) suspended in dry DMF (20 mL), K_2CO_3 (0.9 g, 6.5 mmol) and 2,4-dinitrochlorobenzene (DNCB; 1.9 g, 6.5 mmol) were added and the mixture kept at 80 °C for 3 h. After filtration, the support was washed with DMF (3×5 mL), DMF/H_2O (1:1, v/v, 3×5 mL), H_2O (3×5 mL), H_2O/MeOH (1:1, v/v, 3×5 mL) and MeOH (3×5 mL) and then dried under reduced pressure to give support **2** (0.76 mmol/g). The reaction yield (94%) was evaluated by detaching 1-(2,4-dinitrophenyl) inosine from a weighed amount of support **2** under acid hydrolytic conditions [TFA/H_2O/DCM (3:2:95, v/v/v, 15 min, r.t)] followed by MeOH washings (collected). Then the solid support **2** (0.10 g, 0.076 mmol) was swollen in DMF and left in contact with ethylenediamine (5.0 mmol) in DMF (1.5 mL) under shaking for 8 h at 50 °C. After filtration and washings with DMF (3×5 mL), DMF/MeOH (1:1, v/v, 3×5 mL) and MeOH (3×5 mL) the obtained support **3** was dried under reduced pressure. The product A4 was obtained treating a weighed amount of support **3** with a 2% TFA solution in anhydrous DCM (v/v, 8 min, r.t.), followed by DMF washings (collected). The crude A4 was purified by preparative HPLC using a C-18 reverse-phase column (Merck, 250 mm × 10 mm, particle size 10 µm) eluted with a linear gradient of CH_3CN in H_2O (from 0% to 100% in 60 min, flow 1.3 mL/min).

In particular, 50 mg of support **3** furnished 16 mg of pure 2′,3′-*O*-(4-hydroxymethyl-(1*R*)-benzyliden)-AICA riboside A4. El. An. Calcd. for $C_{17}H_{20}N_4O_6$: C, 54.25; H, 5.36; N, 14.89. Found: C, 54.28; H, 5.38; N, 14.87. ^{1}H-NMR (400 MHz, CD_3OD) δ 7.55 (d, $J = 8.1$ Hz, 2H, arom.), 7.41 (d, $J = 8.1$ Hz, 2H, arom.), 7.38 (s, 1H, 2-H), 6.04 (s, 1H, CH), 5.90 (d, $J = 3.8$ Hz, 1H, 1′-H), 5.26–5.21 (m, 1H, 2′-H), 5.09–5.04 (m, 1H, 3′-H), 4.64 (s, 2H, CH_2OH), 3.82–3.77 (m, 2H, 5′-$H_{a,b}$). ^{13}C-NMR (100 MHz, CD_3OD) δ 167.8, 143.8, 143.2, 134.9, 129.4, 126.7, 126.4,

112.4, 107.9, 90.7, 85.0, 82.8, 81.9, 63.3, 60.9; UV λ_{max} 268 nm; *m/z* 377.1461 (HRESIMS) ($[M+H]^+$, requires 377.1467).

Cell culture

Cell lines derived from human lung (A549), human liver (HepG2) and murine muscle (C2C12) were obtained from the Bank of Human and Animal Continuous Cell Lines-CEINGE Biotecnologie Avanzate, Naples, Italy. The cells were grown in DMEM (Sigma-Aldrich St. Louis, MO) supplemented with 10% fetal bovine serum (FBS; Lonza Basel, Switzerland) and 1% L-glutamine (Sigma-Aldrich). The cells were grown in the flask at 37 °C in a 5% CO_2 as a monolayer.

Western Blotting analysis

Western Blotting analysis was performed as previously described[14]. Briefly, after 12 h starvation A549, HepG2 and C2C12 cells (5×10^4 cells/4 mL) were treated for 2 h with AICAR, ZMP, A3 and A4 (0.5 and 1.0 mM). After incubation, proteins were extracted and separated as previously described[14]. The membranes were incubated with GAPDH (SantaCruz-Biotechnology, Santa Cruz, CA), pACC; p-AMPKα, AMPKα (Cell-Signaling Technology, Danvers, MA) antibodies according to the manufacturer's instructions. Proteins were transferred to PDVF membranes (Millipore Corporation, Billerica, MA) and probed overnight with the appropriate dilution of primary antibodies: pACC, p-AMPKα, AMPKα polyclonal antibodies (Cell Signalling Technology, Inc., Danvers, MA), according to the manufacturer's instructions. The gel was exposed to high-performance autoradiography film (Amersham Biosciences, Piscataway, NJ), digitalized with a scanner (1200 dpi) and analyzed by densitometry with the Jasc Paint Shop Pro 7.00 software (Corel Corporation, Ottawa, Ontario, Canada). All experiments were performed in triplicate.

MTT colorimetric test: proliferation assay

The 3-[4,5-dimethylthiazol-2-yl]-2,5-dipheniltetrazolium-bromide (MTT) test was performed as previously described[15]. Briefly, after 12 h starvation, A549, HepG2 and C2C12 cell lines (4×10^3 cells/100 µL per well) were incubated with or without AICAR and its derivatives (1 mM). After 24, 48 and 72 h of incubation, the cells were stained with MTT/PBS solution. The experiments were performed two times in triplicate.

Serum stability

Serum stability was performed as previously described[15]. Briefly, 10 mL of a 0.5 mM solution of A3 and A4 derivatives were incubated in human serum/TBS (4:1, v/v) at 37 °C and the hydrolytic effects were analyzed by HPLC injecting 10 µL of solution every 15 min. The products were identified by comparison with authentic samples. The analysis were performed using an HPLC apparatus (Jasco PU-2089, equipped with an UV/Vis detector plus-2075) and using a RP18 column (Nucleosil-100, Macherey-Nagel, 250 mm × 4.60 mm, 10 µ) eluted with an increasing amount of acetonitrile in water (linear gradient from 0% to 100%, v/v, in 60 min, flow rate 0.5 mL/min, wavelength 254 nm).

Statistical analysis

Data are expressed as means ± SD and medians. Two groups were compared with 2-tailed unpaired Student's *t*-test. Multiple comparisons were evaluated by one way analysis of variance (ANOVA). The statistical significance was established at $p < 0.05$.

Results

Synthesis of A3 and A4 derivatives

The AICAR-triacetate A3 was produced using acetic anhydride in pyridine as acetylating agent on AICAR and the structure and purity were ascertained by ^{1}H NMR and HPLC, respectively. The derivative A4 was synthesized by exploiting a solid-phase procedure successfully used in our laboratory to produce several derivatives[13,16,17]. The solid support **1** (Scheme 1), containing the 5′-acetyl-inosine moiety with a loading of 0.87 mmol/g, was activated on the purine base by installing the 2,4-dinitrophenyl group on the N-1 position thus obtaining the support **2**. In fact, this aromatic group is a strong electron-withdrawer and renders the C-2 purine atom very reactive towards the ethylenediamine (EDA), which is a reagent that can degrade the purine to 5-aminoimidazole-4-carboxamide (AICA). The reaction with EDA additionally removed the 5′-acetyl protecting group, furnishing the 2′,3′-*O*-(4-hydroxymethyl-(1*R*)-benzylidene)-AICA riboside **3**. Finally, the acid treatment on the support **3** with trifluoroacetic acid (TFA, 2% in DCM) in anhydrous conditions allowed the release of the 2′,3′-*O*-(4-hydroxymethyl-benzylidene)-AICA riboside A4 in an overall yield of 66% (from support **1**). Starting from 50 mg of support **1**, 16 mg of A4 were obtained after HPLC purification. The structure of A4 was confirmed by ^{1}H NMR and MS analyses. Compound A4 proved to be a pure stereoisomer possessing the R configuration at the benzylidene acetal carbon. In fact, the 2D-NOESY experiments on A4 confirmed that the acetal carbon retains the R configuration present on the native benzylidene-acetal-inosine **1** and that no racemization events occur during the TFA treatment on **3**[18].

AICAR derivatives A3 and A4 activate AMPKα inhibiting ACC

We investigated the effects of A3 and A4 on the phosphorylation status of AMPKα validating the results using AICAR and ZMP as positive controls. Cells were treated with two different amounts of AICAR, ZMP and derivatives (0.5 and 1.0 mM) for 2 h. As shown in Figure 2, both A3 and A4 were able to phosphorylate AMPKα in A549, HepG2 and C2C12 cell lines to almost the same extent as AICAR and ZMP ($p < 0.05$).

To further investigate the activity of the new AICAR derivatives, we also determined the phosphorylation status of ACC. The results obtained show that A3 and A4 treatment is associated with the phosphorylation of ACC probably via AMPKα ($p < 0.05$; Figure 2, panel B and C, respectively). In the A549 cells, we did not detect any signals corresponding to ACC probably due to the sensibility of the methodology or due to the lack of any detectable ACC expression. Our data derive from the comparison between untreated and treated cells and confirm the role of AICAR and ZMP as potent AMPKα activators. Moreover, we have demonstrated that A3 and A4 have a similar behavior because they both stimulate the phosphorylation of AMPKα. Finally, we found an over-phosphorylation of ACC in treated cells, probably associated with p-AMPKα activation.

Cytotoxicity of AICAR, ZMP and A3 and A4 derivatives on A549, HepG2 and C2C12 cell lines

To define the cytotoxic effects of A3 and A4, we incubated these derivatives at 1.0 mM concentration on A549, HepG2 and C2C12 cell lines. We observed that A3 and A4, after 24 h of treatment, did not statistically affect cell viability. Instead, after 48 and 72 h of incubations A3 and A4 reduced cell viability (50–60%). These experiments showed that AICAR, ZMP, A3 and A4 do not induce relevant cytotoxic effects *in vitro* up to 24 h (Figure 3). At 48 and

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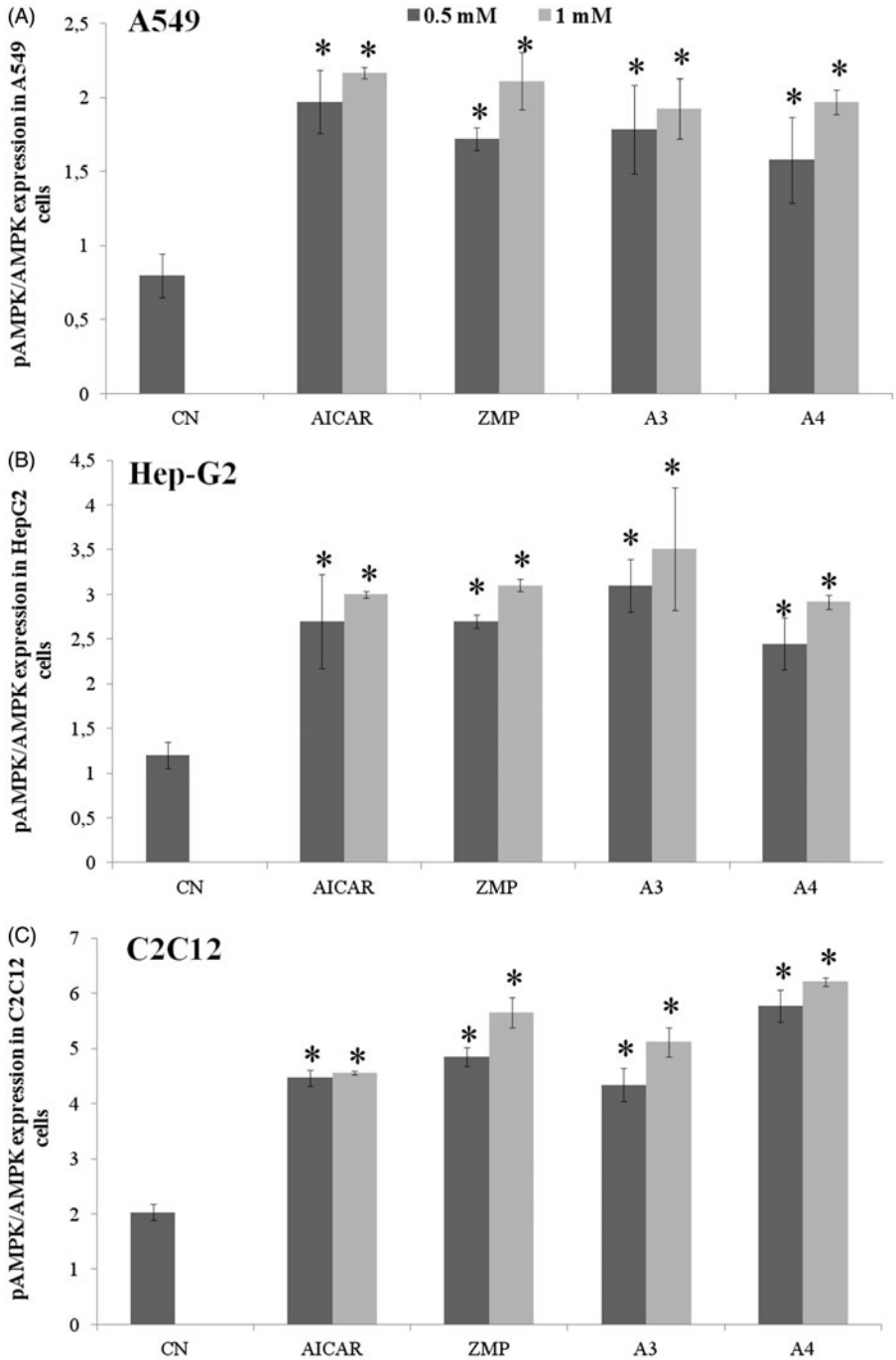

Figure 2. Graphical representation of pixel quantization of p-AMPK in A549, HepG2 and C2C12 cells and one representative WB image of p-AMPK, p-ACC and GAPDH. For other details see materials and methods. WB, western blot. Asterisks indicate statistical differences between control and treatments $p < 0.05$. CN: untreated cells.

72 h, A3 and A4 showed cytotoxic effects to almost the same extent as AICAR and ZMP.

Serum stability

A3 showed solubility in water (pH 7.0) of 1.0 mg/mL, which allows the preparation of its solution in water up to 2.6 mM. As expected, A4 showed a higher solubility in water, which proved to be of 2.0 mg/mL (pH 7.0; up to 5.3 mM solution).

We tested the serum stability of A3 and A4 at 0.5 mM. The results indicated that the acetate functions of A3 were partially hydrolyzed in 15 min furnishing a mixture of diacetate derivatives (5′,2′-, 5′,3′- and 3′,2′-*O*-diacetate) which were completely converted into AICAR in the following 4 h. A4 resulted to be completely stable up to 24 h. A very small amount (2–3%) of AICAR could be detected after 48 h of A4 incubation in serum. The analyses were performed by HPLC comparison with authentic samples.

Discussion

Diabetes and insulin resistance are important public health conditions worldwide and their prevalence continues to increase also in the young population[1]. AICAR plays a central role in the metabolism and in signal transduction increasing insulin properties, up-regulating mitochondrial enzymes in muscles and decreasing intra-abdominal fat[2–4]. It can act as an agonist and antagonist for a number of enzymes crucial for the cell life; among these, AMPKα has a fundamental role in energy homeostasis, being an energy sensor implicated in various

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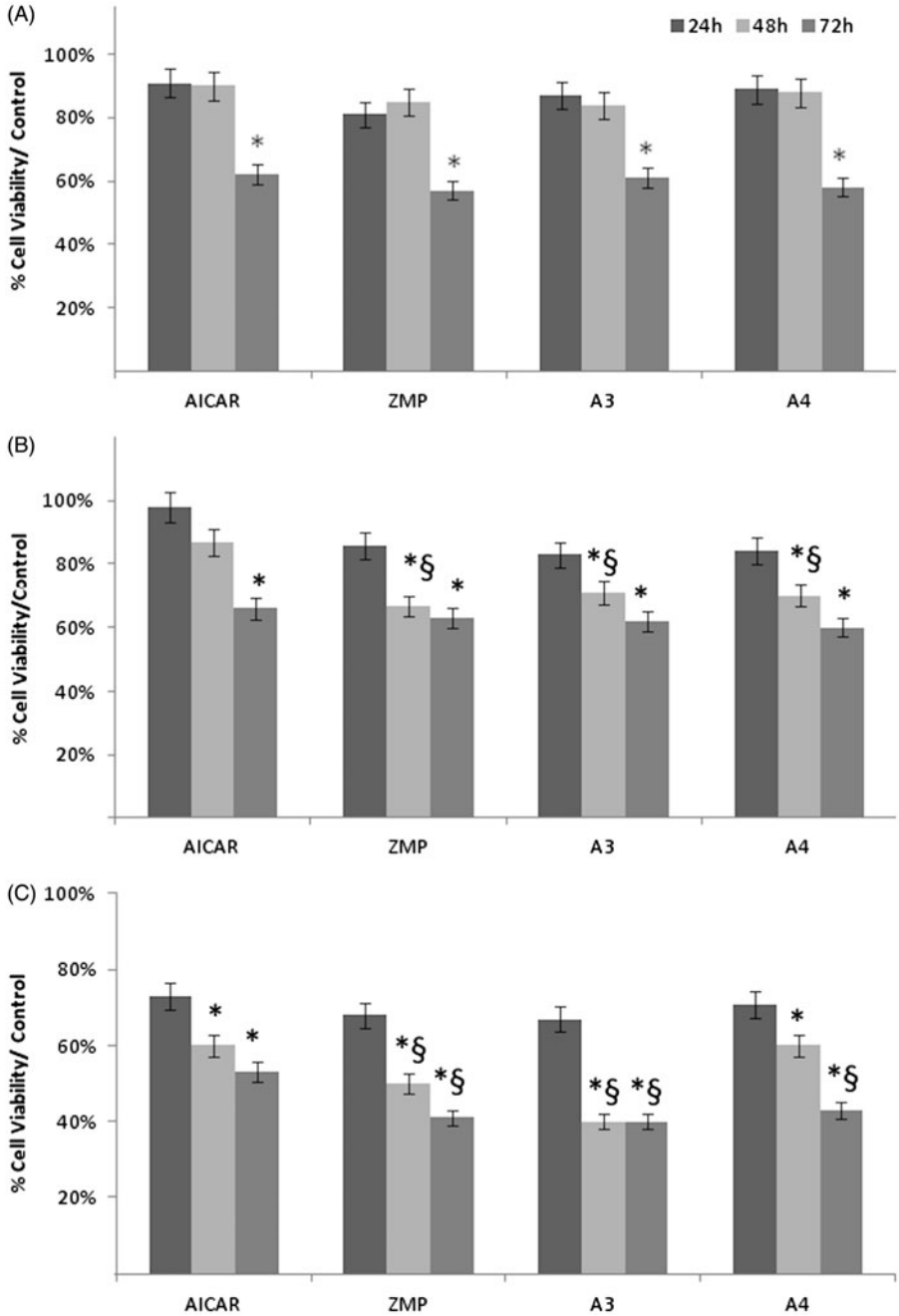

Figure 3. Cytotoxicity of AICAR, ZMP and A3 and A4 derivatives on A549, HepG2 and C2C12 cell lines. (A) A549, (B) HepG2, (C) C2C12 cells were treated with 1.0 mM AICAR, ZMP, A3 and A4 for 24, 48 and 72 h and viability assessed by MTT assay. Values represent means ± SE of experiments performed two times in triplicate. Statistical differences between untreated cells (control) and treated cells are indicated by * and among cells treated with AICAR and derivatives by §. $p < 0.05$.

metabolic diseases such as type 2 diabetes and obesity[19]; in addition, recently AMPKα has been associated with the development of cancer and neurological disorders[20].

In this context, we have investigated the metabolic activities of A3 and A4 newly synthesized AICAR analogs. The former, 2′,3′,5′-tri-*O*-acetyl-AICAR (A3), contains a triacetate ribose moiety. This kind of modified sugar has been proposed and investigated in other nucleosides as a lipophilic pro-ribose moiety[21]. The latter, AICAR derivative A4, contains a 2′,3′-*O*-4-hydroxymethyl-(1*R*)-benzylidene group and conserves its 5′-hydroxy function free. This ribose profile leads to a molecule having lipophilicity that is intermediate between the more polar AICAR and the less polar A3. In particular, we have verified whether A3 and A4 could exert similar or higher phosphorylation activity towards AMPKα than AICAR and ZMP on A549, HepG2 and C2C12 cell lines at two different concentrations (0.5 and 1.0 mM). In addition, we have evaluated the relationship between AMPKα phosphorylation and ACC to verify the efficacy of the treatments. Our findings have shown that the activity of the new molecules A3 and A4 was comparable to that of AICAR and ZMP. In fact, both derivatives were able to phosphorylate both AMPKα and ACC; this phosphorylation occurs probably via AMPKα. Consistent with our results, Rattan et al. have evidenced that AICAR induces a consistent increase of AMPKα and ACC phosphorylation in glioma and prostate cell lines[22] while Kim et al. have achieved a similar result in myotube cells[23]. Given the efficacy of A3 and A4 to activate AMPKα and inhibit ACC, we have verified their potential cytotoxic effects on A549, HepG2 and C2C12 cell lines. Our findings (Figure 3) have indicated that the exposure of the cells to AICAR, ZMP and A3 and A4 (1.0 mM) up to 24 h, does not affect the cell viability. A decrease in cell viability occurs after 48 h (almost 50%). Consistent with our results, Peyton et al. have found that AICAR reduces the cell viability of endothelial cells after just 24 h and Guan et al. have found similar results in CaSki cells[24,25]. Interestingly, in terms of cell proliferation, we have tested ERK1/2 phosphorylation: according to the MTT results, at 2 h of incubation, ERK1/2 phosphorylation was down regulated in

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treated cells (data not shown). These findings indicate that A3 and A4 are molecules with promising therapeutic properties for the treatment of the metabolic disorders. Consistent with our results, Baumann et al. in myeloma cells have found an increase on p-AMPKα and a decrease of p-ERK1/2 phosphorylation together with a reduction of cell viability induced by AICAR after 90 min of treatment[26]. On the other hand, in contrast with our results, Kim et al. have found that AICAR induces p-ERK1/2 phosphorylation after 15 min of treatment until 60 min in osteoblastic cell lines[27].

Conclusions

In conclusion, the need to improve therapeutic strategies against metabolic disorders has prompted the discovery of new molecules of metabolic processes able to reduce insulin resistance. In this study, we synthesized two novel AICAR derivatives with: (a) modified ribose moieties that impart to them different cell permeability and serum stability; (b) consistent metabolic activity in terms of AMPK and ACC phosphorylation; (c) low-grade cytotoxic activity toward human and mouse cell lines.

Altogether, the two analogs could be considered as promising potential therapeutic tools.

Further studies are needed to completely elucidate the activity of A3 and A4 and the possibility of their use for the treatment of metabolic disorders.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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