The Adenosine Monophosphate (AMP) Analog, 5-Aminoimidazole-4-Carboxamide Ribonucleotide (AICAR) Inhibits Hepatosteatosis and Liver Tumorigenesis in a High-Fat Diet Murine Model Treated with Diethylnitrosamine (DEN)

Jianlong Gao*
Rui Xiong*
Dan Xiong
Wenxing Zhao
Sheng Zhang
Tao Yin
Xinhua Zhang
Guozhen Jiang
Zhenyu Yin

* These authors contributed equally to this work

Corresponding Author: Zhenyu Yin, e-mail: zhengyuyinqd@sina.com

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Background: The development and progression of hepatocellular carcinoma (HCC) are associated with obesity and hepatosteatosis. AMP-activated protein kinase (AMPK) regulates metabolic homeostasis. This study aimed to investigate the effects of treatment with the adenosine monophosphate (AMP) analog, 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) on hepatosteatosis in a mouse model fed a high-fat diet (HFD), and on hepatocellular carcinoma (HCC) induced by diethylnitrosamine (DEN) in the HFD mouse model.

Material/Methods: Male C57BL/6 male mice from two weeks of age were fed a high-fat diet, resulting in hepatosteatosis. HFD mice (15–20 per group) were treated with AICAR and without AICAR. HFD mice were treated with DEN, with and without AICAR. Mouse liver tissues were examined histologically using lipid histochemical stains, immunohistochemistry, and immunofluorescence. Levels of cytokines, alanine transaminase (ALT), triacylglyceride (TAG), and apoptosis were determined. Western blot was used to detect AMPK, pAMPK, STAT3, and pSTAT3. Real-time polymerase chain reaction (RT-PCR) detected expression of the ACL, FAS, CD36, ATGL, CPT1, and IL6 genes.

Results: In the HFD mouse model, AICAR treatment inhibited hepatic lipid synthesis and IL-6 expression. In the DEN-treated mice, AICAR treatment reduced tumorigenesis, IL-6 signaling, and STAT3 activation. Short-term AICAR treatment had no significant effect in advanced HCC.

Conclusions: In an HFD mouse model, treatment with AICAR reduced the development of hepatosteatosis, and following treatment with the liver carcinogen, DEN, AICAR reduced the development of HCC. These preliminary findings support further studies on the role of AICAR in fatty liver disease and HCC.

MeSH Keywords: 2,5-Dimethoxy-4-Methylamphetamine • AMP-Activated Protein Kinases • Carcinoma, Hepatocellular

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Hepatocellular carcinoma (HCC) is the most common primary malignancy of the liver, and is associated with risk factors that include infection with hepatitis B virus (HBV) and hepatitis C virus (HCV), alcoholic liver disease (ALD), and non-alcoholic liver disease (NALD) that includes hepatosteatosis, and non-alcoholic steatohepatitis (NASH) [1–3]. Many of the risk factors for HCC are associated with inflammation. In hepatosteatosis, a condition in which fat accumulates in the liver, a common underlying factor is obesity [4,5]. Epidemiological studies have shown that increased body mass and obesity increase the risk of several types of cancer [6]. HCC that is associated with obesity has been shown to be associated with increased levels of inflammatory cytokines, including interleukin-6 (IL-6) and tumor necrosis factor alpha (TNFα), which activate the oncogenic transcription factor, signal transducer and activator of transcription 3 (STAT3), and trigger tumorigenesis [7]. Therefore, control of body weight and prevention of obesity, and metabolic syndrome may reduce the risk of liver cancer.

In eukaryotic cells, AMP-activated protein kinase (AMPK) is a sensor of energy and nutrient status and is a master regulator of metabolic homeostasis by switching off anabolic pathways, including synthesis of fatty acids, triglycerides, cholesterol and proteins [8–10]. Therefore, AMPK is a good pharmacological target for the treatment of metabolic diseases such as obesity, type 2 diabetes and cardiovascular disease [11]. At the same time, AMPK is also a potential cancer therapeutic target [12], because its activation arrests cell cycle, presumably by suppressing most of the metabolic changes that occur in rapidly proliferating cells. However, whether AMPK should be activated or inhibited during cancer therapy remains an open question that requires further investigation. However, AMPK activation during energy stress has been shown to enhance the survival of cancer cells [13]. On the other hand, AMPK has been found to negatively regulate aerobic glycolysis in cancer cells, and suppress tumor growth in vivo [14].

The adenosine monophosphate (AMP) analog, 5-aminomidazole-4-carboxamide ribonucleotide (AICAR), is a membrane-permeable prodrug that activates AMPK [15]. In cells, AICAR is metabolized to the nucleotide, 5-aminimidazole-4-carboxamide ribonucleotide (ZMP), an AMP analog with similar effects on the AMPK complex [16], including allosteric activation, AMPK alpha 2 (T172) phosphorylation, and protection from dephosphorylation. Mice fed high-fat diets develop obesity, and were chosen as a model in this study, to evaluate the effects of long-term AICAR treatment on hepatosteatosis chemically triggered HCC in diet-induced obese mice, and treated them with AICAR. Several carcinogenic compounds can be used in mouse models to induce HCC, including diethylnitrosamine (DEN).

The aims of this study were to investigate the effects of treatment with the adenosine monophosphate (AMP) analog, 5-aminimidazole-4-carboxamide ribonucleotide (AICAR), on hepatosteatosis and in a mouse model induced with diethylnitrosamine (DEN) in the HFD mouse model.

### Material and Methods

#### Mouse model and chemical induction of liver tumors

Male C57BL/6 male mice from two weeks of age were given a single intraperitoneal (i.p.) injection of diethylnitrosamine (DEN) (25 mg/kg) (Sigma) to induce hepatocellular carcinoma (HCC). Mice were randomly divided into phosphate-buffered saline (PBS)-treated or 5-aminimidazole-4-carboxamide ribonucleotide (AICAR)-treated groups (15–20 per group). Commencing at six weeks of age, and continuing until the end of the study, mice were fed a high-fat diet (HFD) consisting of 60% fat, 20% carbohydrate, and 20% protein (D12492) (Research Diets, New Brunswick, NJ, USA). Every other day, the HFD mice received i.p. injections of PBS or AICAR (350 mg/kg) (Toronto Research Chemicals Inc., Toronto, Canada). Tumors in the liver were counted and measured by nuclear magnetic resonance (NMR). At the end of the study, after the mice were euthanized, the tumors were also counted and measured with a caliper, and liver tissues were immediately sampled for frozen section, biochemical, histological and immunohistochemical analysis. Mouse studies were conducted in the Laboratory Animal Center at Xiamen University.

#### Histological, immunohistochemical, and immunofluorescence assays

Samples of mouse liver tissue were fixed in 10% formalin for 24–48 h. For histological examination, paraffin-embedded liver tissues were sectioned onto glass slides and stained with hematoxylin-eosin (H&E) and Masson’s trichrome stain. For immunohistochemical staining, anti-interleukin (IL)-6 antibody (Abcam, Cambridge MA, USA) was used as primary antibody and biotinylated goat anti-rabbit IgG as the secondary antibody. For immunofluorescence staining, paraffin-embedded liver tissues were stained with terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) (Clontech) reagent and probed with anti-5-bromo-2’-deoxyuridine (BrdU) antibody (#5292) (Cell Signaling Technology) respectively. Frozen tissue sections were stained with Oil Red O (ORO) to visualize the tissue lipid content on light microscopy.
**Cytokines and ALT levels in mouse serum**

IL-6, tumor necrosis factor (TNF)-α and alanine aminotransferase (ALT) in serum were determined using kits from Nanjing Jiancheng Bioengineering Institute (Nanjing, China), according to the manufacturer’s instructions.

**Measurement of triacylglyceride (TAG)**

Serum and liver triacylglyceride (TAG) were measured using LabAssay™ Triglyceride colorimetric-enzymatic assay (290-63701) (Wako Pure Chemical Industries, Ltd., Osaka, Japan), according to the manufacturer’s instructions.

**Western blot**

Liver samples were homogenized in lysis buffer (pH 7.5), containing 20 mM Tris-HCl, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM ethylene glycol tetraacetic acid (EGTA), 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate, and protease inhibitor. Equal amounts of liver homogenate (25 μg of total protein) were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to polyvinylidene difluoride (PVDF) membranes. After blocking with 5% dried skimmed milk powder for 1 hour at room temperature, the membranes were probed with antibodies to AMP-activated protein kinase (AMPK), pAMPK, signal transducer and activator of transcription 3 (STAT3) (Proteintech), pSTAT3 (Cell Signaling), β-actin (Sigma) overnight at 4°C. The membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 2 hours at room temperature. The proteins were detected using an ECL Plus kit (Beyotime, Shanghai, China).

**RNA isolation and real-time polymerase chain reaction (RT-PCR)**

Total RNA was extracted with Trizol reagent (Invitrogen) and used as a template to prepare cDNA using M-MLV RTase system (Takara). Gene expression was quantified using SYBR Green (Takara). Gene expression was quantified using SYBR Green PCR System (Applied Biosystems). The primer sequences used (Takara). Gene expression was quantified using SYBR Green PCR System (Applied Biosystems). The primer sequences used were as follows:

- Carnitine palmitoyltransferase (CPT1), forward 5'-CTCCGCCTGATCCGACTCATCGTACTCC-3' and reverse 5'-GCCGGACTCATCGTACTCC-3'.
- IL-6, forward 5'-TCTACACCTAAGAGCTCGGA-3' and reverse 5'-GAATTGCCATTGCACAACTCTTT-3'.
- β-actin, forward 5'-GTGACGTTGACATCCGTAAAGA-3' and reverse 5'-GCCGGACTCATCGTACTCC-3'.
- β-actin served as an internal control.

**Statistical analysis**

Student’s t-test was used to test statistical significance between groups. Data were presented as the mean ±SEM. A p-value <0.05 was considered to be statistically significant.

**Results**

**AICAR treatment inhibited diethylnitrosamine (DEN)-induced liver tumorigenesis in the high-fat diet (HFD) mouse model**

To examine whether AICAR could reduce the risk of hepato-cellular carcinoma (HCC), the two-week-old male mice were given a single intraperitoneal (i.p.) injection of diethylnitrosamine (DEN) (25 mg/kg). Four weeks later, at six weeks of age, the mice fed the mice with the high-fat diet (HFD) and were treated with AICAR (350 mg/kg) once every other day (Figure 1A, protocol 1). Nuclear magnetic resonance (NMR) imaging showed that AICAR-treated mice developed significantly HCC nodules at eight months when compared with control mice, treated with PBS (Figure 1B). At 10 months of age, mice were treated with AICAR developed strikingly fewer HCCs per liver (Figure 1C, left panel). The number of tumors, as well as the size of the largest tumors, was significantly reduced (Figure 1C). Also, the relative liver weight decreased significantly following AICAR treatment (Figure 1C). Relative liver weight was the weight of the liver normalized to body weight. AICAR-treated mice gained less weight when compared with control mice (Figure 1D). Therefore, in this HFD mouse model, AICAR reduced DEN-induced hepatocarcinogenesis.

**Short-term AICAR treatment showed no significant effect in advanced hepatocellular carcinoma (HCC) in the HFD mouse model**

To investigate whether AICAR had a therapeutic benefit in advanced HCC, treatment was given at 32 weeks, at which point the mice had already developed HCC nodules (Figure 2A, protocol 2). The treatment lasted for the next two months. Tumor size was not significantly reduced after a further month of treatment (Figure 2B). AICAR treatment over two months did not significantly decrease the number of tumors, the weight of the largest tumor, and the relative weight of the liver (Figure 2C).
Figure 1. AICAR inhibited diethylnitrosamine (DEN)-induced hepatocarcinogenesis in the high-fat diet (HFD) mouse model. (A) Male C57BL/6 mice were injected intraperitoneal with diethylnitrosamine (DEN) (25 mg/kg) two weeks of age and maintained on a high-fat diet (HFD) from week 6 to week 40. Four weeks after diethylnitrosamine DEN treatment, 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) (350 mg/kg) was administered every other day until the mice were euthanized. (B) Representative nuclear magnetic resonance (NMR) images at eight months show the reduced size of liver tumors in DEN-treated HFD mice treated with AICAR. (C) Representative images and measurement of the liver tumors showed that AICAR reduced the number of tumors, the weight of the largest tumor, and the relative weight of the liver tumors. Arrows indicate the hepatocellular carcinoma (HCC) tumors in the mice. Results are shown as the mean ±SEM (n=10–11). * p<0.05, ** p<0.01, *** p<0.001. (D) Gain in body weight in the control and AICAR-treated HFD mice. * p<0.05.
Figure 2. AICAR showed no significant effect in advanced hepatocellular carcinoma (HCC) in the high-fat diet (HFD) mouse model. (A) Male C57BL/6 mice were given diethylnitrosamine (DEN) (25 mg/kg) at two weeks of age and maintained on a high-fat diet (HFD) from week 6 to week 40. AICAR (350 mg/kg) was given every other day from week 32 to week 40. (B) Representative nuclear magnetic resonance (NMR) images show that AICAR treatment for one month did not significantly alter the size of liver tumors. (C) AICAR administration over two months did not significantly decrease the number of tumors, the weight of the largest tumor, and the relative weight of the liver. Results are shown as the mean ±SEM (n=10–11).
Therefore, short-term treatment with AICAR in the HFD mouse model did not confer therapeutic benefits in late-stage HCC.

**AICAR treatment prevented hepatosteatosis induced by a high-fat diet (HFD) in the mouse model**

Using histological analysis, we investigated whether AICAR could prevent HFD-induced hepatosteatosis, which is known to be a risk factor for the development of liver tumors [17]. In the mice fed an HFD, light microscopy with hematoxylin and eosin (H&E) staining showed marked ballooning in the mouse liver cells, indicating lipid content (Figure 3A). This result was supported by Oil Red O (ORO) staining of frozen sections, which also showed that AICAR treatment reduced the volume of lipid droplets in the liver cells (Figure 3B). Triacylglycerol (TAG) levels in both the mouse liver and serum decreased with AICAR administration (Figure 3C, 3D). Masson’s trichrome staining showed reduced amounts of collagen fibers in AICAR-treated mice (Figure 3E), indicating that liver fibrosis was suppressed.

Also, mRNA levels of the citrate cleavage enzyme gene, ACL, and the fatty acid synthase gene, FAS, which are both involved in lipid synthesis, were reduced in AICAR treated mice (Figure 3F). However, AICAR did not affect mRNA expression levels of CD36, ATGL, and CPT1, which are involved in lipid uptake, oxidation, and lipolysis, respectively (Figure 3F). These results showed that AICAR suppressed hepatosteatosis, which could be attributed to reduced lipogenesis.

**AICAR treatment prevented liver damage and inhibited liver cell proliferation in the HFD mouse model**

A previously published study showed that HCC in obese mice had more proliferating cells and reduced apoptotic cell death when compared with HCC in lean mice [18]. Therefore, in this study, we examined whether AICAR treatment could support these previous findings. Mice in protocol 1, at six months of age, received an injection of 5-bromo-2’-deoxyuridine (BrdU), two hours before they were euthanized. Liver sections were immediately collected and formalin-fixed. Paraffin-embedded liver tissues were then stained with terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) reagent and then probed with an anti-BrdU antibody. AICAR treatment significantly reduced the rate of apoptotic cell death (Figure 4A) and reduced compensatory proliferation in mouse livers with HCC (Figure 4B).

**AICAR treatment inhibited the expression of IL-6 and STAT3 phosphorylation in the HFD mouse model**

Obesity has been previously reported to be associated with the production of the inflammatory cytokines, interleukin (IL)-6 and tumor necrosis factor (TNF)-α, which are both critical to obesity-promoted onset and development of HCC [19]. In this study, IL-6 levels were significantly reduced in AICAR-treated HFD mice (Figure 5A), and AICAR also reduced the levels of IL-6 mRNA and protein in the liver (Figure 5B, 5C). The levels of TNF-α were also reduced, although this finding did not reach statistical significance (Figure 5A). Using alanine transaminase (ALT) as a marker of liver damage, AICAR treatment reduced ALT levels, indicating a degree of protection of the liver from injury (Figure 5D).

As shown in Figure 2, short-term treatment with AICAR did not affect the progression of late-stage HCC, suggesting that AICAR inhibited liver tumorigenesis through a chronic, long-term process. Comparison of the amount of IL-6 expressed in mice at three months and six months was made (Figure 5E). At three months, control and AICAR mice had similar body weight (Figure 1D), liver lipid content (Figure 5F, left panel), and liver IL-6 levels (Figure 5E, left panel). However, at six months of age, AICAR-treated mice had lower body weight (Figure 1C), and had reduced levels of lipids (Figure 5F, right panel) and IL-6 in the liver, when compared with controls (Figure 5E, right panel). These results indicated that AICAR reduced lipid accumulation in the liver via a chronic process that ultimately results in suppressed IL-6 production.

Western blotting showed the effect of AICAR on the phosphorylation of STAT3, which is a downstream target of IL-6 signaling in the liver. Mice at three or six months of age were euthanized 1 hour after injection with phosphate-buffered saline (PBS) or AICAR. Liver sections were collected promptly, and snap-frozen in liquid nitrogen. Liver homogenates were then subjected to Western blotting (Figure 6A). AICAR treatment increased the phosphorylation of AMPK and diminished phosphorylation of STAT3 in the livers at three months and at six months, respectively (Figure 6A).

Also, AICAR was shown to mitigate the effects of diethylnitrosamine (DEN) in vivo (Figure 6B), by analyzing mice that were euthanized 1 hour after injection with DEN (100 mg/kg) and AICAR. DEN treatment induced rapid production of IL-6 and phosphorylation of STAT3 [20]. However, AICAR abrogated DEN-induced phosphorylation of STAT3 (Figure 6B). Therefore, AICAR inhibited DEN-induced cytokine production and signaling, presumably through its AMP-like effects on AMPK. This finding of suppression of IL-6-dependent inflammation in the mouse liver has been previously reported [21].

**Discussion**

The aims of this study were to investigate the effects of treatment with the adenosine monophosphate (AMP) analog, 5-aminomidazole-4-carboxamide ribonucleotide (AICAR) on
Figure 3. AICAR prevented high-fat diet (HFD)-induced hepatic steatosis in the HFD mouse model. Hepatocellular carcinoma (HCC)-bearing (protocol 1) male mice underwent histological and biochemical analysis at 10 months of age. (A) Representative liver sections stained with hematoxylin and eosin (H&E) from control and AICAR-treated mice. Magnification: left panel, ×100; right panel, ×200. (B) Frozen liver sections stained with Oil Red O (ORO). Magnification: left panel, ×100; right panel, ×200. Long-term AICAR administration reduced both liver (C) and serum (D) triacylglycerides (TAG). * p<0.05. (E) The extent of hepatic fibrosis was determined histologically by detection of collagen staining with Masson’s trichrome stain. (F) Relative mRNA levels of enzymes involved in lipid metabolism, as determined by real-time polymerase chain reaction (RT-PCR). Results are shown as the mean ±SEM (n=5–6). * p <0.05.
hepatosteatosis in a mouse model fed a high-fat diet (HFD), and on hepatocellular carcinoma (HCC) induced by diethylnitrosamine (DEN) in the HFD mouse model. The findings of this study showed that long-term AICAR treatment prevented hepatic steatosis, reduced the production of the inflammatory cytokine, interleukin (IL)-6, and inhibited the development of HCC in the HFD mouse model. Also, AICAR treatment suppressed phosphorylation of the oncogenic transcription factor, signal transducer and activator of transcription 3 (STAT3). The results of this study showed that AMP-activated protein kinase (AMPK) should be studied further as a potential biomarker for HCC and that further, long-term studies should be undertaken on the effects of AICAR on hepatosteatosis and obesity-related HCC.

In the mouse model used in this study, the HFD promoted hepatic steatosis in DEN-treated mice and promoted the onset and progression of tumorigenesis in the mouse liver. Therefore, reducing hepatic steatosis may prevent liver cancer. A previously published study showed that mice deficient in heat shock transcription factor 1 (HSF1) had decreased chronic hepatic steatosis and dramatically reduced tumorigenesis following DEN administration [22]. Metformin also protects mice from chemically-induced liver tumors by inhibiting pathways that drive hepatic lipogenesis [23]. However, the protective effect of metformin is believed to be independent of AMPK activation [24]. There is evidence from the published literature that AMPK agonists represent a class of promising agents that could prevent hepatic steatosis, by activating AMPK in vivo to regulate lipid metabolism in the liver, including resveratrol, a natural polyphenol found in red wine, and S17834, a synthetic polyphenol [25]. Salicylate, a plant product, has also been shown to directly activate AMPK and increase fat utilization in mice [13]. These compounds require further study.

**Figure 4.** AICAR administration prevented liver damage and inhibited compensatory proliferation in the liver in the high-fat diet (HFD) mouse model. (A) AICAR reduced apoptotic cell death, as measured by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining. (B) AICAR prevented cell proliferation in six-month-old mice that were pulse-labeled with 5-bromo-2’-deoxyuridine (BrdU), 2 hours before being euthanized. Liver sections were stained with antibodies to BrdU.
Figure 5. AICAR inhibited interleukin (IL)-6, alanine transaminase (ALT), and lipid levels in the liver in the high-fat diet (HFD) mouse model. The hepatocellular carcinoma (HCC)-bearing mice (protocol 1) underwent additional histological and biochemical analysis at 10 months of age (A–D). (A) Levels of cytokines in the serum, as measured by enzyme-linked immunosorbent assay (ELISA). Bars represent the mean ±SEM (n=10–11). *** p<0.001. (B) Liver interleukin (IL-6) mRNA levels decreased in AICAR treated mice. Bars represent the mean ±SEM (n=10–11). * p<0.05. (C) IL-6 staining of liver sections from HCC-bearing mice at 10 months of age. (D) Levels of alanine transaminase (ALT) in serum, as measured by ELISA. (E) IL-6 staining of liver sections from mice three and six months of age. (F) Hepatic lipid staining of liver sections from mice three and six months of age.
Figure 6. AICAR suppressed STAT3 phosphorylation in the liver in the high-fat diet (HFD) mouse model. (A) Western blotting of indicated proteins expression in the liver of mice at three or six months of age, which were euthanized 1 hour after AICAR injection. (B) Western blotting of indicated proteins expression in the liver of mice at three or six months of age, which were euthanized 1 hour after injection with diethylnitrosamine (DEN) (100 mg/kg) and AICAR.

but might be considered as future anti-cancer drugs for the treatment of HCC.

In the present study, AICAR, a membrane-permeable AMPK agonist, was shown for the first time to prevent lipid accumulation in the liver and to suppress the effects of the chemically induced development of HCC. This study was the first demonstration of the effects of AICAR on primary HCC in vivo and is supported by a previous in vitro that demonstrated the inhibition of cancer cell growth and tumor development in vivo and in xenograft models [13]. Furthermore, the findings of this study showed that AICAR reduced lipid accumulation in the liver in the HFD mouse model via a chronic process that was associated with reduced expression of IL-6, which is an important risk indicator, which has previously been shown to be correlated with poor prognosis in HCC [26]. Also, IL-6 expression has been reported to be increased in HCC progenitor cells, and the progression of liver cells to HCC depends on the autocrine effects of IL-6 [27].

The findings of this study showed that AICAR prevented hepatic steatosis, reduced IL-6 production, and inhibited DEN-induced hepatocarcinogenesis in vivo. The prodrug, AICAR, also inhibited STAT3 phosphorylation. Although AMPK was activated with AICAR treatment, this may not be the only mechanism through which AICAR affects the development of HCC. However, a short-term AICAR regimen has no significant effects on advanced HCC. Therefore, although AICAR might not be suitable to treat late-stage HCC, the findings of this study have shown AICAR might have a potential role in the prevention or treatment of HCC or other types of malignancy, and that AMPK requires future study as a potential anti-cancer drug target.

Conclusions

In a mouse model fed a high-fat diet (HFD), when compared with an untreated control group, treatment with the adenosine monophosphate (AMP) analog, 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR), reduced the development of hepatosteatosis. In the HFD mouse model treated with the liver carcinogen, diethylnitrosamine (DEN), AICAR treatment reduced the development of hepatocellular carcinoma (HCC). These preliminary findings in an HFD mouse model support the need for further studies on the role of AICAR in fatty liver disease and HCC.

Conflict of interest

None.

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