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Combination therapy with 5-amino-4-imidazolecarboxamide riboside and arsenic trioxide in acute myeloid leukemia cells involving AMPK/TSC2/mTOR pathway

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Received June 5, 2012, accepted July 6, 2012

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Pharmazie 68: 117–123 (2013) *doi: 10.1691/ph.2013.2666*

The aim of this study was to demonstrate the effects of the AMP-activated protein kinase (AMPK) activator 5-amino-4-imidazolecarboxamide riboside (AICAR) in combination with arsenic trioxide (ATO) in acute myeloid leukemia cells and determine its mechanism of action. Cell lines were either exposed to each drug alone or both the drugs simultaneously. Cell proliferation, cell cycle and apoptosis were assessed. Combination index (CI) method was used to calculate the synergistic, additive, or antagonistic effects of these drugs. Western blot technique was used to study the signaling molecules in the AMPK/TSC2/mTOR pathway. Simultaneous exposure of HL-60 cells to AICAR and ATO indicated a synergism (CI < 1), whereas CI on NB4 cells was greater than 1. In HL-60, the change in expression level of each protein was quite significant in the presence of the combination as compared to that induced through any single agent. On the contrary, ATO weakened the effect of AICAR-mediated AMPK activation in NB4 cells. ATO caused a profound decrease in the protein level of PML/RARα in NB4 cells after 48 h, but there was no change with AICAR and the combination. The combination of AICAR and ATO produced a synergistic effect in the treatment of HL-60 cells involving AMPK/TSC2/mTOR pathway, and AICAR reduced ATO-mediated apoptotic death on acute promyelocytic leukemia NB4 cells.

1. Introduction

Acute myeloid leukemia (AML) is a clinically and genetically hematological malignant disease, which accounts for 15–20% of childhood leukemia. Its long-term survival rate is 45%-56% (Kaspers and Creutzig 2005).

Arsenic trioxide (As_2O_3, ATO) can effectively induce apoptosis in acute promyelocytic leukemia (APL) cells *in vivo* and *in vitro* (Bachleitner-Hofmann et al. 2002; Miller et al. 2002; Momeny et al. 2010). In fact, several clinical trials have been conducted to determine the efficacy of ATO in treating several conditions, such as AML, multiple myeloma, non-Hodgkin' s lymphoma, and chronic lymphocytic leukemia cells (Lu et al. 2003; Berenson and Yeh 2006; Merkel et al. 2008; Biswas et al. 2010). The molecular mechanism through which ATO prevents the proliferation of tumor cells includes the following processes: i) induction of programmed cell death, ii) inhibition of cell growth and proliferation, iii) hindering angiogenesis, iv) stimulating cell differentiation, v) stemming tumor cell invasion, and vi) metastasis (Miller et al. 2002; Wei et al. 2005; Carney 2008). Although treatment with ATO cures clinical remission in patients with APL without severe toxicity, relapse with ATO-resistant cells still occurs (Jing et al. 1999; Au et al. 2000; Takahashi 2010). In fact, ATO is a toxic substance at relatively high exposures (Ratnaike 2003). Thus, a chemopreventive approach incorporating the use of other active drugs to increase the efficacy of ATO has been recommended. This lowers its dosage of action and is considered as one of the alternative strategies used in the management of AML.

Recently, AMP-activated protein kinase (AMPK) was reported to be involved in cell growth regulation. In fact, it was identified as a potential target for cancer (Luo et al. 2005). When activated, AMPK stimulates tuberous sclerosis complex (TSC) through phosphorylation of TSC2/tuberin protein, which acts as a GTPase-activating protein for the small GTPase Rheb. Although we have not been able to completely elucidate the molecular mechanism underlying mTOR activation by GTP, it has been proved that TSC2 activation switches off Rheb, resulting in the inhibition of mTOR activity (Inoki et al. 2003). Research studies have found that AMPK activation induces apoptosis in several types of cancerous cells, such as human prostate cancer (Xiang et al. 2004), renal cell carcinoma (Woodard et al. 2010), breast cancer (Hadad et al. 2008), gastric cancer cells (Saitoh et al. 2004), pancreatic cells (Kefas et al. 2003), and hepatic carcinoma cells (Meisse et al. 2002). Thus, the fact that AMPK activators can serve as potential antineoplastic agents has drawn quite a lot of attention.

5-Amino-4-imidazolecarboxamide riboside (AICAR) is an AMPK agonist. AICAR has been used extensively in research studies to define the roles of AMPK signaling in various cellular processes. Clinical studies have found that AICAR inhibits the proliferation of several cancer cell lines, such as C6 glioma

cells, T98 G astrocytoma cells, MCF-7 breast cancer cells, PC-3 prostate carcinoma cells, and hematological cancer cells (Xiang et al. 2004; Rattan et al. 2005). In hematological malignancies, clinical studies have reported that AICAR induces cell cycle arrest and apoptotic cell death in ALL cells (Sengupta et al. 2007). Moreover, combination of AICAR with the mTOR inhibitor rapamycin further enhanced the suppression of cell proliferation (Sengupta et al. 2007). AMPK seems to be an attractive target in mantle cell lymphoma cells (Drakos et al. 2009). More recently, clinical trials conducted on nude mice indicated that the AMPK activator metformin had potent inhibitory effects on various AML cell lines, primary AML cells, and AML xenografts, which has been associated with decreased mTOR signaling (Green et al. 2010). These research studies have raised the potential of targeting the AMPK/TSC/mTOR pathway in the treatment of AML.

In an earlier experiment, we found that the cell permeable nucleoside AICAR inhibits proliferation and induces apoptosis in acute myeloid leukemia HL-60 and NB4 cells through AMPK activation. Moreover, the cytotoxic efficacy of ATO and AICAR combination has not been assessed in these types of cancer cells. Thus, with the purpose of evaluating whether the combination of ATO and AICAR would be effective in treating patients with acute myeloid leukemia, we incubated HL-60 and NB4 leukemic cells with ATO (at clinically achievable concentrations) in combination with AICAR. In this manner, we evaluated a possible additive/synergistic anti-proliferative and/or pro-apoptotic activity.

2. Investigations and results

2.1. Cell growth inhibition with AICAR or ATO

The inhibition of proliferation caused by AICAR and ATO in the HL-60 and NB4 cell lines was assessed after being exposed to drugs for the following time intervals: 24, 48, and 72 h. Growth of these two cell lines was inhibited *in vitro* in a time- and concentration-dependent manner. As shown in Fig. 1A and Table 1, the NB4 cell line was sensitive to low concentrations of AICAR. The maximum effect of AICAR was obtained in NB4 cell (IC50 556.41 ± 34.49 μmol/L, 443.96 ± 12.14 μmol/L and 296.15 ± 7.10 μmol/L for 24, 48, and 72 h, respectively). Moreover, AICAR also showed a time- and concentration-dependent effect on HL-60 cell, as outlined by the calculated IC50 after 24, 48 and 72 h exposures (1885.50 ± 56.38 μmol/L, 1367.10 ± 2.38 μmol/L and 907.95 ± 9.59 μmol/L, respectively). In comparative terms, however, the resulting impact was much higher than those found in NB4 cell. The IC50 values for ATO were 6.52 ± 0.26 μmol/L and 3.28 ± 0.01 μmol/L for 48 h in the HL-60 and NB4 cell lines, respectively. As compared to NB4, HL-60 seemed to be more resistant to the two selected drugs.

2.2. Combinatorial effects of AICAR and ATO

To obtain the data for mathematical analysis of the interaction between two drugs, HL-60 and NB4 cells were either treated with various concentrations of each drug alone and in combination. Then, the cell viability was assessed by the CCK-8 test (Fig. 1B). Surprisingly, when both drugs were applied together, AICAR sharply reduced ATO cytotoxicity towards NB4 cells. On the other hand, AICAR increased ATO-mediated killing of HL-60 cells.

Then, the data was analyzed by the method of Chou and Talalay (1984), based on the assumptions that the two drugs were either mutually exclusive or mutually non-exclusive. The CI values were plotted against the values of the affected fractions (Fig. 1C). Simultaneous exposure of HL-60 cells to different concentrations of AICAR and ATO for 24, 48, and 72 h indicated a synergism ($CI < 1$, Fig. 1A and Fig. 1C). Fig. 1C shows the CI/Fraction affected (F_a) curve of HL-60 cells exposed to AICAR and ATO with the simultaneous schedule of treatment. However, as shown by the CI/F_a curve in Fig. 1C, the simultaneous treatments of AICAR and ATO were antagonistically active on NB4 cell proliferation.

2.3. The combination of AICAR and ATO inhibited cell cycle progression

Effects on cell cycle were studied using flow cytometry so as to further characterize the effects of the combination of AICAR and ATO. Thus, we assessed the changes in the G1, S, and G2 phases of the cell cycle with either agent, and compared them with the controls (Fig. 2A). Single agent AICAR significantly decreased the number of cells at both the G1 and G2 phases of the cell cycle, but AICAR increased the number of cells at the S phase when measured at 48 h. On the other hand, single agent ATO induced G2 phase arrest in acute myeloid leukemia cells. While comparing the two cell lines, the effects on cell cycle were mainly derived from the single agent AICAR. However, G2 phase indicated a significant decrease, when it was treated with the combination containing AICAR.

2.4. Induction of apoptosis by AICAR, ATO or the combination

Effects on apoptotic cell death were studied by flow cytometry using the medium containing Annexin V. The antagonistic characteristics showed detectable apoptosis, which was caused by ATO at 2 μmol/L after 48 h of treatment, and AICAR at 250 μmol/L or 750 μmol/L (Fig. 2B and 2C). As compared with the control groups, the total number of apoptotic cells, which included both early- and late-stage cells, was significantly higher in each of the drug-treated groups ($P < 0.05$; Fig. 2C). In case of HL-60 cells, when compared with any of the single-drug treatment groups (AICAR, 33.69%; ATO,16.89%; $P < 0.05$), the number of apoptotic cells was significantly greater in the groups treated with the drug combination (40.64%). On the contrary, AICAR diminished the proportion of both early (annexin$^+$/PI$^-$) and late apoptotic cells (annexin$^+$/PI$^+$) in ATO-exposed NB4 cell cultures (29.56% apoptotic cells were reported in the presence of both agents, compared with 20.22% and 51.82% in the presence of AICAR or ATO alone, respectively). These results were in agreement with the findings of cell growth inhibition studies.

2.5. Western blot analysis of signal transduction changes with the combination of AICAR and ATO

Next, we analyzed AMPK/TSC2/mTOR signaling pathway, which was blocked or induced by either drug treatment. Fig. 3A illustrates the summary of key findings, which focused on the 48-h time point in HL-60 and NB4 cell lines. At this time point, the AMPK agonist, AICAR enhanced the protein expressions of p-AMPKα and TSC2. Also, it suppressed p-mTOR, but it did not induce any change on either total AMPKα or total mTOR of the two cell lines. ATO decreased both the phosphorylation level of AMPKα and mTOR in HL-60 cells, but markedly inhibited TSC2 in NB4. In HL-60, the change in expression level of each protein was quite significant in the presence of the combination as compared to that induced through any single agent. On the contrary, ATO weakened the effect of AICAR-mediated AMPK activation.

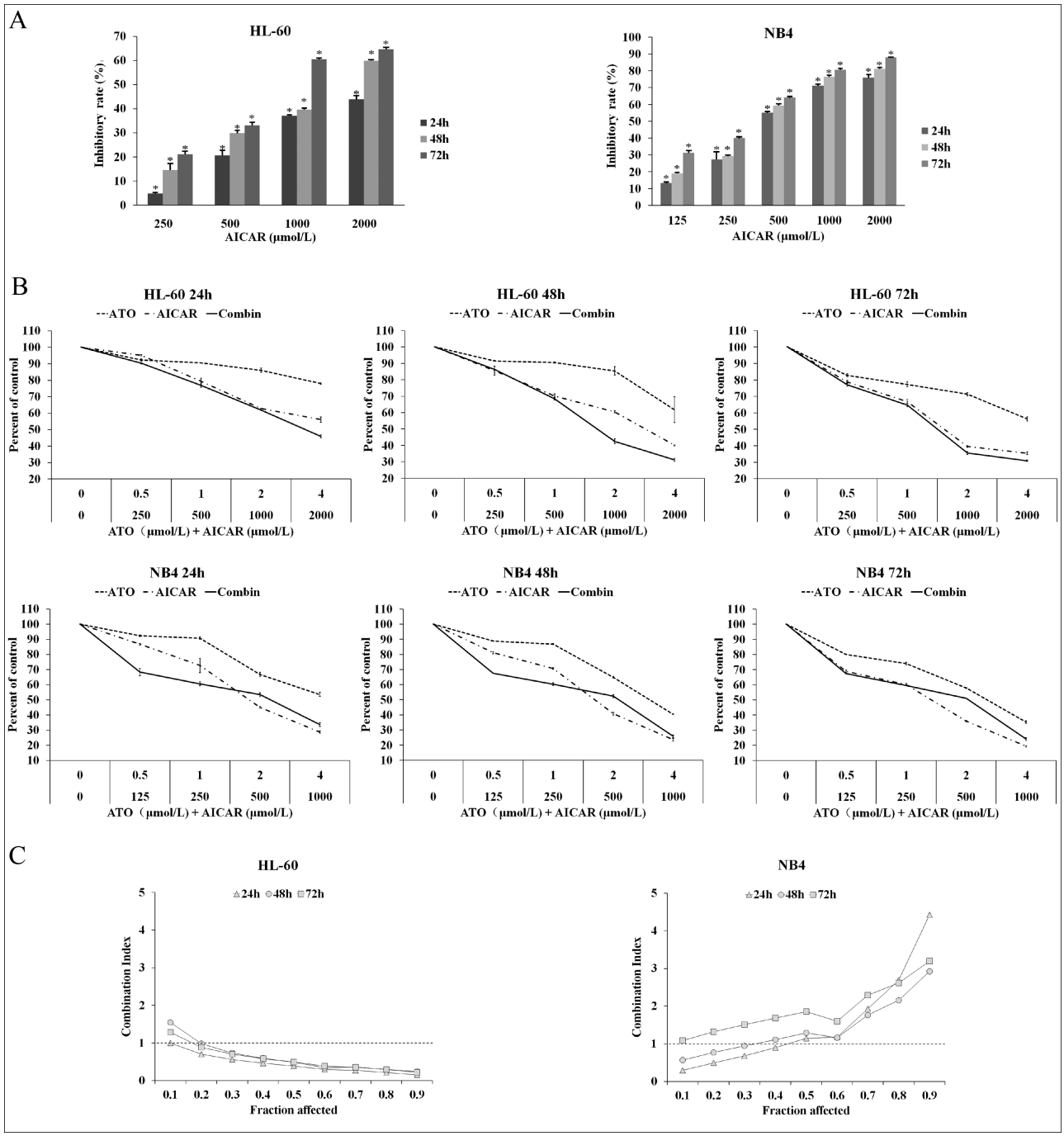

Fig. 1: Effects of 5-Amino-4-imidazolecarboxamide riboside (AICAR) alone and in combination with arsenic trioxide (ATO) on HL-60 and NB4 for 24, 48 and 72 h. (A) Curve of growth inhibitory rate of HL-60 and NB4 cells after treatment with AICAR. Symbols and bars, mean values and SE, respectively. * $P < 0.001$ compared with control. (B) The anti-proliferative effects of drugs were studied on HL-60 and NB4 cell lines. Symbols and bars, mean values and SE, respectively. (C) The effects of a combination of ATO and AICAR on HL-60 and NB4. CI = 1 indicates an additive effect, CI < 1 indicates a synergistic effect and CI > 1 indicates an antagonistic effect

Table 1: IC_{50} values (μmol/L) of ATO and AICAR in HL-60 and NB4 cells

Cell line	HL-60			NB4		
	24 h	48 h	72 h	24 h	48 h	72 h
ATO	20.85 ± 1.46	6.52 ± 0.26	6.20 ± 0.21	5.50 ± 0.17	3.28 ± 0.01	2.14 ± 0.02
AICAR	1885.50 ± 56.38	1367.10 ± 2.38	907.95 ± 9.59	556.41 ± 34.49	443.96 ± 12.14	296.15 ± 7.10

Note: arsenic trioxide (ATO), 5-Amino-4-imidazolecarboxamide riboside (AICAR).

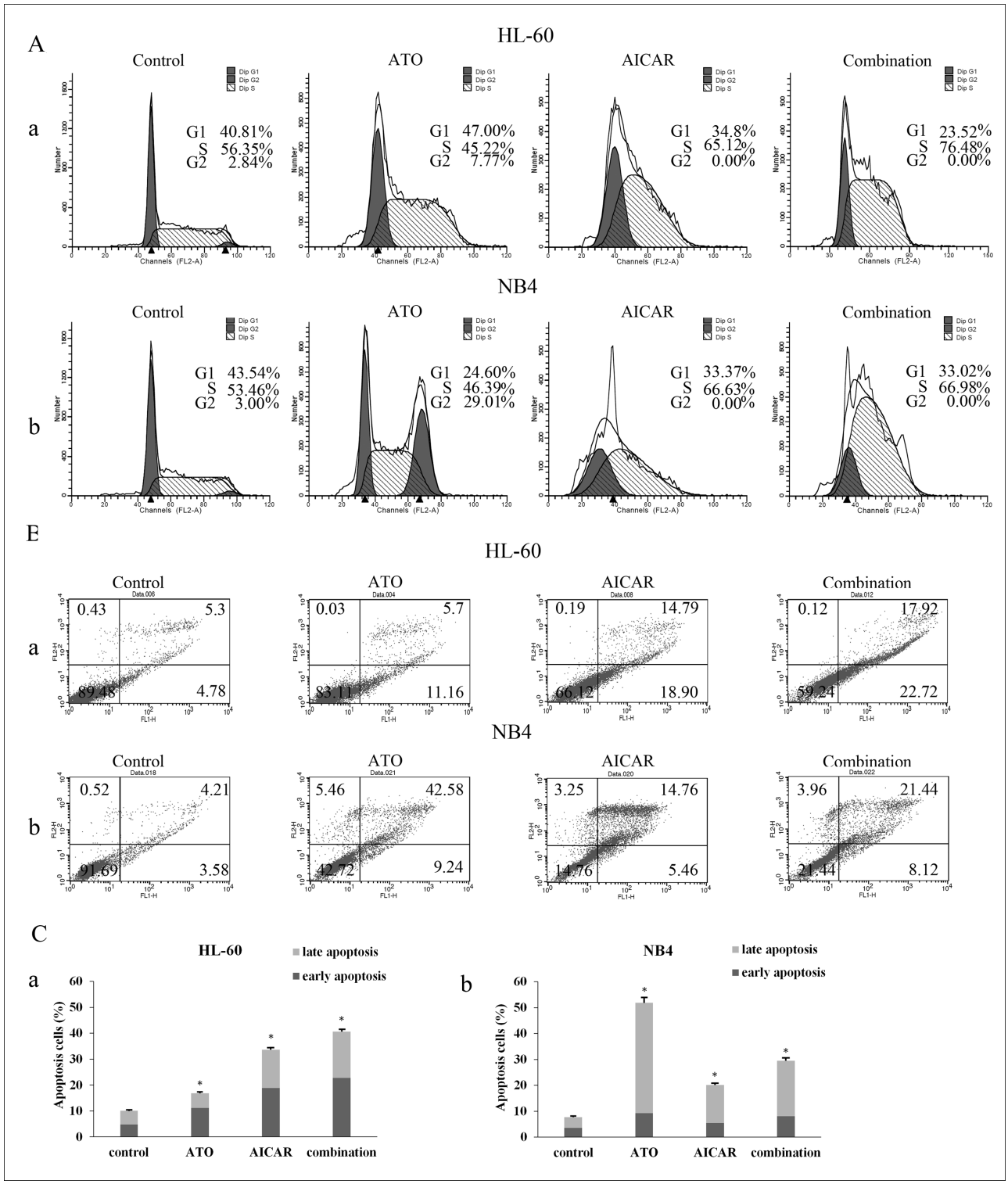

Fig. 2: Effects of single agent and combination therapy on cell cycle and apoptosis. (A) Flow cytometry analysis of cell cycle in HL-60 and NB4 cells. (B) The pro-apoptotic effects in the HL-60 and NB4 cells. (C) Early and late apoptotic cells after being subjected to 48-h treatment with the indicated agents. * $P < 0.05$ compared with control

2.6. Effect of PML/RARα protein expression by AICAR, ATO or both in NB4 cells

In this study, we investigated whether ATO in combination with AICAR induced an antagonistic effects in acute promyelocytic leukemia NB4 cells. So, we examined the protein level of PML/RARα, which was identified as a dominant-negative translocation product, etiologic for APL (de The et al. 1991; Chen et al. 1996). As shown in Fig. 3B, ATO caused a profound decrease in the protein level of PML/RARα in NB4 cells after 48 h such that we could not detect its expression by Western blot. However, there was no change in NB4 cells with AICAR and combination.

3. Discussion

In this study, we explored how the combinations of ATO with AICAR created an effective impact on HL-60 and NB4: the two selected cell lines of acute myeloid leukemia. In this study,

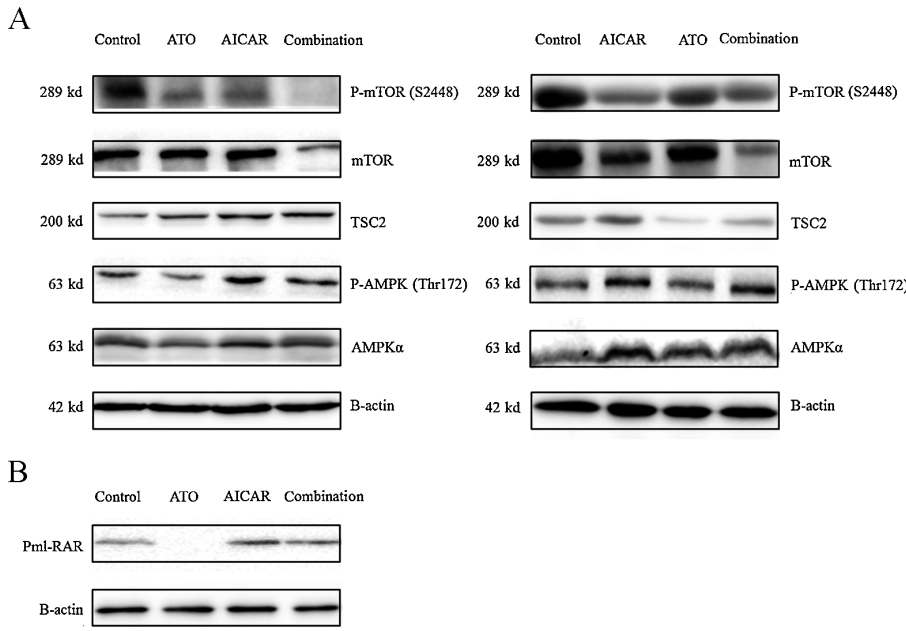

Fig. 3: Western blot analysis of AMPK-mTOR pathway and PML/RARA fusion protein expression. (A) Expression of AMPK-mTOR pathway with arsenic trioxide (ATO), 5-amino-4-imidazolecarboxamide riboside (AICAR) and the combination in HL-60 and NB4 cells. (B) Expression of PML/RARA fusion protein with ATO, AICAR and both for 48 h on NB4. Actin protein levels were measured to serve as an internal control

we found that both AICAR and ATO produced potent cytotoxic effects when incubated with acute myeloid leukemia HL-60 and NB4 cell lines. Although these effects were apparent when each drug was incubated separately with the cell lines, the cytotoxic effects were more pronounced and significant when these drugs were incubated simultaneously in HL-60 cells. This is the first study that illustrates such a synergistic effect induced by AICAR and ATO in non-APL AML cell lines. In addition, it also indicates that the combination of AICAR and ATO might be quite effective in treating patients with non-APL AML. However, the findings of this study also suggest that the ATO in combination with AICAR has antagonistic effects on APL NB4 cells. This suggests that the addition of AICAR is unlikely to benefit patients with APL, who are first treated with the single agent ATO.

As a cellular energy sensor, AMPK has recently been found to be a crucial regulator of cell growth, proliferation, and apoptosis. AICAR is extensively used as an activator of AMPK. It is transported within the cells through the adenosine transporter and is phosphorylated by adenosine kinase to form ZMP, which activates AMPK. The exact mechanism through which AICAR activates AMPK is controversial so far. Also, whether AMPK is the primary therapeutic target of AICAR continues to be a matter of debate. Research (Guo et al. 2009) with AICAR showed that the anti-growth properties of this AMPK activator are not fully mediated through inhibition of mTORC1 signaling. Rather, this is achieved through inhibition of cholesterol and fatty acid synthesis by inhibiting ACC and HMG-CoA. Moreover, its AMPK-independent effects have also been reported (Xiang et al. 2004). In our study, AICAR mediates its effect via activation of AMPK (Fig. 3A). AICAR alone down-regulates the phosphorylation of mTOR level, while increasing AMPK phosphorylation and TSC2 in both HL-60 and NB4 cell lines. The AICAR treatment is effective in inhibiting the proliferation of both HL-60 and NB4 cells.

Many clinical trials involving ATO have been conducted to determine its ability in treating several hematologic malignancies, such as refractory AML, myelodysplastic syndrome, non-Hodgkin lymphoma, chronic lymphocytic or chronic myelogenous leukemia, acute T cell leukemia, and multiple myeloma (Ravandi 2004). The effects of ATO on cell signaling pathways have been extensively studied, although the results of these research studies are often controversial. For instance, ATO was occasionally observed to stimulate JNK and PI3K/Akt activation and down-regulate ERK in some leukemia cell models (Shim et al. 2002; Redondo-Munoz et al. 2010). Earlier studies have suggested that arsenic inhibited neurite outgrowth by inhibiting the LKB1-AMPK signaling pathway, and ATO activated mTOR by down-regulating TSC2, a negative upstream effector of mTOR (Altman et al. 2008; Wang et al. 2010). Our data show that ATO can slightly down-regulate AMPKα phosphorylation and TSC2 levels, but it suppresses the mTOR activation. We presume that it refers to independent TSC2 manner.

For mathematical analysis, we had to obtain the data associated with the interaction between the two drugs. So, HL-60 and NB4 cells were treated with various concentrations of each drug alone and in combination. The data shows that simultaneous exposure of HL-60 cells to different concentrations of AICAR and ATO created a synergistic effect ($CI < 1$). AICAR increases ATO-mediated killing of HL-60 cells. Then, we explored changes in phosphor-protein levels in order to understand the differential effects of this combination in acute myeloid leukemia cell lines. According to our study, after being treated with AICAR, a decrease in mTOR phosphorylation correlated well with an increase in the phosphorylation of AMPKα. Also, TSC2 level was also slightly enhanced at 48 h. Furthermore, in case of HL-60 cells showing highest sensitivity to the combination, p-mTOR decrease was reported after being exposed to ATO. Therefore, we attribute the synergistic effects induced by ATO and AICAR in HL-60 cells to a more significant blockade of mTOR signaling.

Our experimental results indicate that AICAR markedly reduces ATO cytotoxicity towards NB4 cells. Meanwhile, AICAR increases ATO-mediated killing of HL-60 cells. These data indicates that AICAR antagonizes the *in vitro* anticancer activity of ATO in a cell-specific manner. The acute myeloid leukemia cell lines, HL-60 (AML M2) and NB4 (AML M3), are responsive to ATO treatment. Unlike HL-60 cells, NB4 cells carry the translocation t (15;17) with the resultant chimeric protein PML/RARα (Dalton et al. 1988; Chen et al. 1996). According to a previous report, ATO-induced relocalization of PML-RARα onto

nuclear bodies and the subsequent degradation of PML/RARα are pivotal in inducing the apoptosis of NB4 cells (Chen et al. 1996). With the purpose of evaluating whether AICAR reduces ATO-mediated cytotoxic effects on NB4 through PML/RARα, we examined the protein levels of PML/RARα. Surprisingly, AICAR invalidates PML/RARα degradation. This explains the antagonistic interactions inhibiting cell proliferation and inducing apoptosis. Further studies are still needed to elucidate this phenomenon.

In summary, the combination of AICAR and ATO can produce a synergistic effect in the treatment of HL-60 cells involving AMPK/TSC2/mTOR pathway. Also, AICAR reduces ATO-mediated apoptotic death on APL NB4 cells. A significant pro-apoptotic effect was also observed when the two drugs were used simultaneously, but further studies are required to identify and elucidate the additional mechanisms responsible for this synergism. Nevertheless, our present results may help in designing clinical trials to further evaluate the combination therapy of AICAR and ATO in non-APL AML patients.

4. Experimental

4.1. Reagents

AICAR was purchased from Sigma (St Louis, MO). Arsenic trioxide (ATO) was purchased from Pharmaceuticals Limited Company of Harbin Medical University (Heilongjiang, China). The cell counting kit-8 (CCK-8 kit), the HRP-labeled secondary antibody, SDS Lysis Buffer, phenylmethanesulfonyl fluoride (PMSF), BCA protein assay kit and BeyoECL Plus were all purchased from Beyotime (Jiangsu, China). FITC Annexin V Apoptosis Detection Kit was obtained from BD Biosciences (Pharmingen, Becton Dickinson Co., CA, USA). The mTOR rabbit mAb was obtained from Cell Signaling Technology (Beverly, MA, USA). The antibodies against TSC2, p-AMPKα (Thr 172), AMPKα and β–actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

4.2. Cell culture and treatment

The human leukemia HL-60 (M2, PML/RARα negative) and NB4 (M3, PML/RARα positive) cells were obtained from Cell Bank, Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). These cells were maintained as an asynchronous, exponentially growing population in RPMI-1640 medium (Hyclone, Logan, UT), which was supplemented with 10% fetal bovine serum (Hyclone), 100 U/ml penicillin, 100 μg/ml streptomycin (Sigma Chemical Co.) and 2 mmol/L L-glutamine at 37 °C in an atmosphere of 5% CO2. The cells used in these experiments were in their logarithmic phase of growth, and their viability was greater than 90%.

The AICAR stock solution (40 mmol/L in RPMI-1640 medium) was prepared and diluted to working concentration, immediately before being used. ATO was prepared as an 8 mmol/L stock solution in RPMI-1640 medium. The cells were treated with 0.5, 1, 2, and 4 μmol/L of ATO according to pharmacokinetic data (Shen et al. 1997). In subsequent studies, we conducted an elaborate characterization of the cell response to a combination of 2 μmol/L ATO and AICAR, which was used in concentration of IC_{35} (250 μmol/L for NB4, 750 μmol/L for HL-60).

4.3. Cell proliferation assays

Cell viability was assessed with the help of CCK-8 kit. HL-60 and NB4 cell lines were grown to mid log phase cultures, which were diluted to 5×10^4 cells/mL and treated for 24, 48 and 72 h, either with each drug alone or in combination. Control cells were treated with RPMI-1640 medium alone. Then, CCK-8 solution was added to each sample, followed by incubation at 37 °C for 3 h. Supernatant was transferred to a 96-well plate. The optical density (OD) was determined at 450 nm using a microplate reader (Tecan Group Ltd, Infinite M200, Switzerland). In each sample, three parallel experimental groups were used to assess the cell viability. Furthermore, each experiment was performed in triplicate.

4.4. Examination of the effect of the drug combination

Before testing the combined effect of two agents, the individual IC_{50} values were determined from a single-agent exposure of the cells to either ATO or AICAR, using the CCK-8 assay. Then, the combination index (CI) was determined according to the median-effect method of Chou and Talalay (1984). CI > 1, CI = 1, and CI < 1 indicated antagonism, additive effect, or synergism, respectively.

4.5. Cell cycle analysis

Cells were treated with ATO (2 μmol/L, IC_{25} for HL-60, IC_{35} for NB4), AICAR (IC_{35}), or both for 48 h. HL-60 and NB4 cells were washed twice with cold phosphate-buffered saline (PBS), and then fixed in 70% ethanol at –20 °C overnight. Ethanol-fixed cells were resuspended in 0.5 mL of 50 μg/mL propidium iodide (PI) containing the following reagents: 100 μg/ml RNase, 0.1% NP-40, and 0.1% sodium citrate. Then, the cells were incubated on ice for 30 min. The cell cycle distribution was evaluated from 20000 cells using the FACS caliber (Becton Dickinson, USA) and the ModFit LTTM software (Becton Dickinson, USA).

4.6. Apoptosis analysis

For detecting the percentage of apoptotic cell by Annexin-V/PI, after incubation with ATO (2 μmol/L, IC_{25} for HL-60, IC_{35} for NB4), AICAR (IC_{35}), or both for 48 h, the HL-60 and NB4 cells were harvested and washed with cold PBS twice. Then, these cells were resuspended in 100 μL binding buffer at a concentration of 1×10^6 cells/mL. Then, the cells were incubated in 5 μL Annexin-V–fluorescein isothiocyanate (FITC) and 5 μL PI in the dark for 15 min. In total, 400 μL binding buffer was then added in each tube, prior to being analyzed with flow cytometer. Cells that stain positive for FITC Annexin V and negative for PI are undergoing early apoptosis. Cells that stain positive for both FITC Annexin V and PI imply the following possibilities: i) cells are at the end stage of apoptosis, ii) cells are undergoing necrosis, or iii) cells are already dead. Cells that stain negative for both FITC Annexin V and PI are alive. Such cells are not undergoing measurable apoptosis.

4.7. Western Blot analysis

The leukemia NB4 and HL-60 cells culture with treated with ATO (2 μmol/L, IC_{25} for HL-60, IC_{35} for NB4), AICAR (IC_{35}), or both for 48 h. Then, the cells were suspended in lysate buffer containing the following reagents: 50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 10 mmol/L sodium pyrophosphate, 50 mmol/L NaF, 1 mmol/L sodium vandate, 0.1% SDS, 10% glycerol, 1% NP40, 0.5% sodium deoxycholate, 1 μmol/L leupeptin, 0.1 μmol/L aprotinin, and 1 mmol/L PMSF. Before immunoblotting, the protein concentrations were determined with a BCA detection kit. Then, they were adjusted to equal concentrations across different samples. The samples (40 μg) were loaded and separated by 8% or 12% SDS-PAGE gel. Then, these samples were transferred to PVDF membranes. According to the supplier's protocol, the membranes were probed with various antibodies. Afterwards, signal was measured by an ECL kit and relative photographic density was quantitated by Odyssey Fc Imaging System.

4.8. Statistical analysis

All data were expressed in terms of mean ± standard deviation. The statistical significance of the differences was conducted by ANOVA followed by a Student's t-test, wherein $P < 0.05$ represented significance.

Acknowledgements: We thank Lei Yue (School of Life Science and Technology, Harbin Institute of Technology, Harbin, China) for providing assistance in flow cytometer analysis. The experiments in this study were performed by Yang Yu (Laboratory of Medical Genetics, Harbin Medical University, Harbin, China), under the guidance of Dr. Zheng (School of Life Science and Technology, Harbin Institute of Technology, Harbin, China).

References

Altman JK, Yoon P, Katsoulidis E, Kroczynska B, Sassano A, Redig AJ, Glaser H, Jordan A, Tallman MS, Hay N, Platanias LC (2008) Regulatory effects of mammalian target of rapamycin-mediated signals in the generation of arsenic trioxide responses. J Biol Chem 283: 1992–2001.

Au WY, Ma SK, Ooi C, Liang R, Kwong YL (2000) Unusual manifestations of acute leukemia. Case 1. CNS extramedullary relapse of acute promyelocytic leukemia after arsenic trioxide-induced remission. J Clin Oncol 18: 3435–3437.

Bachleitner-Hofmann T, Kees M, Gisslinger H (2002) Arsenic trioxide: acute promyelocytic leukemia and beyond. Leuk. Lymphoma 43: 1535–1540.

Berenson JR, Yeh HS (2006) Arsenic compounds in the treatment of multiple myeloma: a new role for a historical remedy. Clin Lymphoma Myeloma 7: 192–198.

Biswas S, Zhao X, Mone AP, Mo X, Vargo M, Jarjoura D, Byrd JC, Muthusamy N (2010) Arsenic trioxide and ascorbic acid demonstrate promising activity against primary human CLL cells *in vitro*. Leuk Res 34: 925–931.

Carney DA (2008) Arsenic trioxide mechanisms of action–looking beyond acute promyelocytic leukemia. Leuk Lymphoma 49: 1846–1851.

Chen GQ, Zhu J, Shi XG, Ni JH, Zhong HJ, Si GY, Jin XL, Tang W, Li XS, Xong SM, Shen ZX, Sun GL, Ma J, Zhang P, Zhang TD, Gazin C, Naoe T, Chen SJ, Wang ZY, Chen Z (1996) *In vitro* studies on cellular and molecular mechanisms of arsenic trioxide (As2O3) in the treatment of acute promyelocytic leukemia: As2O3 induces NB4 cell apoptosis with downregulation of Bcl-2 expression and modulation of PML-RAR alpha/PML proteins. Blood 88: 1052–1061.

Chou TC, Talalay P (1984) Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv. Enzyme Regul 22: 27–55.

Dalton WT Jr., Ahearn MJ, McCredie KB, Freireich EJ, Stass SA, Trujillo JM (1988) HL-60 cell line was derived from a patient with FAB-M2 and not FAB-M3. Blood 71: 242–247.

de The H, Lavau C, Marchio A, Chomienne C, Degos L, Dejean A (1991) The PML-RAR alpha fusion mRNA generated by the t(15;17) translocation in acute promyelocytic leukemia encodes a functionally altered RAR. Cell 66: 675–684.

Drakos E, Atsaves V, Li J, Leventaki V, Andreeff M, Medeiros LJ, Rassidakis GZ (2009) Stabilization and activation of p53 downregulates mTOR signaling through AMPK in mantle cell lymphoma. Leukemia 23: 784–790.

Green AS, Chapuis N, Maciel TT, Willems L, Lambert M, Arnoult C, Boyer O, Bardet V, Park S, Foretz M, Viollet B, Ifrah N, Dreyfus F, Hermine O, Moura IC, Lacombe C, Mayeux P, Bouscary D, Tamburini J (2010) The LKB1/AMPK signaling pathway has tumor suppressor activity in acute myeloid leukemia through the repression of mTOR-dependent oncogenic mRNA translation. Blood 116: 4262–4273.

Guo D, Hildebrandt IJ, Prins RM, Soto H, Mazzotta MM, Dang J, Czernin J, Shyy JY, Watson AD, Phelps M, Radu CG, Cloughesy TF, Mischel PS (2009) The AMPK agonist AICAR inhibits the growth of EGFRvIII-expressing glioblastomas by inhibiting lipogenesis. Proc Natl Acad Sci USA 106: 12932–12937.

Hadad SM, Fleming S, Thompson AM (2008) Targeting AMPK: a new therapeutic opportunity in breast cancer. Crit Rev Oncol Hematol 67: 1–7.

Inoki K, Zhu T, Guan KL (2003) TSC2 mediates cellular energy response to control cell growth and survival. Cell 115: 577–590.

Jing Y, Dai J, Chalmers-Redman RM, Tatton WG, Waxman S (1999) Arsenic trioxide selectively induces acute promyelocytic leukemia cell apoptosis via a hydrogen peroxide-dependent pathway. Blood 94: 2102–2111.

Kaspers GJ, Creutzig U (2005) Pediatric acute myeloid leukemia: international progress and future directions. Leukemia 19: 2025–2029.

Kefas BA, Heimberg H, Vaulont S, Meisse D, Hue L, Pipeleers D, Van de Casteele M (2003) AICA-riboside induces apoptosis of pancreatic beta cells through stimulation of AMP-activated protein kinase. Diabetologia 46: 250–254.

Lu D, Bai XC, Gui L, Li M, Zheng WS, Han XQ, Luo SQ (2003) Arsenic trioxide-induced apoptosis of human malignant lymphoma cell lines and its mechanisms. Di Yi Jun Yi Da Xue Xue Bao 23: 997–1001.

Luo Z, Saha AK, Xiang X, Ruderman NB (2005) AMPK, the metabolic syndrome and cancer. Trends Pharmacol Sci 26: 69–76.

Meisse D, Van de Casteele M, Beauloye C, Hainault I, Kefas BA, Rider MH, Foufelle F, Hue L (2002) Sustained activation of AMP-activated protein kinase induces c-Jun N-terminal kinase activation and apoptosis in liver cells. FEBS Lett 526: 38–42.

Merkel O, Heyder C, Asslaber D, Hamacher F, Tinhofer I, Holler C, Stocher M, Prokesch A, Papak C, Scheideler M, Trajanoski Z, Greil R (2008) Arsenic trioxide induces apoptosis preferentially in B-CLL cells of patients with unfavourable prognostic factors including del17p13. J Mol Med 86: 541–552.

Miller WH Jr., Schipper HM, Lee JS, Singer J, Waxman S (2002) Mechanisms of action of arsenic trioxide. Cancer Res. 62: 3893–3903.

Momeny M, Zakidizaji M, Ghasemi R, Dehpour AR, Rahimi-Balaei M, Abdolazimi Y, Ghavamzadeh A, Alimoghaddam K, Ghaffari SH (2010) Arsenic trioxide induces apoptosis in NB-4, an acute promyelocytic leukemia cell line, through up-regulation of p73 via suppression of nuclear factor kappa B-mediated inhibition of p73 transcription and prevention of NF-kappaB-mediated induction of XIAP, cIAP2, BCL-XL and survivin. Med Oncol 27: 833–842.

Ratnaike RN (2003) Acute and chronic arsenic toxicity. Postgrad Med J 79: 391–396.

Rattan R, Giri S, Singh AK, Singh I (2005) 5-Aminoimidazole-4-carboxamide- 1-beta-D-ribofuranoside inhibits cancer cell proliferation *in vitro* and *in vivo* via AMP-activated protein kinase. J Biol Chem 280: 39582–39593.

Ravandi F (2004) Arsenic trioxide: expanding roles for an ancient drug? Leukemia 18: 1457–1459.

Redondo-Munoz J, Escobar-Diaz E, Hernandez Del Cerro M, Pandiella A, Terol MJ, Garcia-Marco JA, Garcia-Pardo A (2010) Induction of B-chronic lymphocytic leukemia cell apoptosis by arsenic trioxide involves suppression of the phosphoinositide 3-kinase/Akt survival pathway via c-jun-NH2 terminal kinase activation and PTEN upregulation. Clin Cancer Res 16: 4382–4391.

Saitoh M, Nagai K, Nakagawa K, Yamamura T, Yamamoto S, Nishizaki T (2004) Adenosine induces apoptosis in the human gastric cancer cells via an intrinsic pathway relevant to activation of AMP-activated protein kinase. Biochem. Pharmacol 67: 2005–2011.

Sengupta TK, Leclerc GM, Hsieh-Kinser TT, Leclerc GJ, Singh I, Barredo JC (2007) Cytotoxic effect of 5-aminoimidazole-4-carboxamide-1-beta-4-ribofuranoside (AICAR) on childhood acute lymphoblastic leukemia (ALL) cells: implication for targeted therapy. Mol Cancer 6: 46.

Shen ZX, Chen GQ, Ni JH, Li XS, Xiong SM, Qiu QY, Zhu J, Tang W, Sun GL Yang KQ, Chen Y, Zhou L, Fang ZW, Wang YT, Ma J, Zhang P, Zhang TD, Chen SJ, Chen Z, Wang ZY (1997) Use of arsenic trioxide (As2O3) in the treatment of acute promyelocytic leukemia (APL): II. Clinical efficacy and pharmacokinetics in relapsed patients. Blood 89: 3354–3360.

Shim MJ, Kim HJ, Yang SJ, Lee IS, Choi HI, Kim T (2002) Arsenic trioxide induces apoptosis in chronic myelogenous leukemia K562 cells: possible involvement of p38 MAP kinase. J Biochem Mol Biol 35: 377–383.

Takahashi S (2010) Combination therapy with arsenic trioxide for hematological malignancies. Anticancer Agents Med Chem 10: 504–510.

Wang X, Meng D, Chang Q, Pan J, Zhang Z, Chen G, Ke Z, Luo J, Shi X (2010) Arsenic inhibits neurite outgrowth by inhibiting the LKB1-AMPK signaling pathway. Environ Health Perspect 118: 627–634.

Wei LH, Lai KP, Chen CA, Cheng CH, Huang YJ, Chou CH, Kuo ML, Hsieh CY (2005) Arsenic trioxide prevents radiation-enhanced tumor invasiveness and inhibits matrix metalloproteinase-9 through downregulation of nuclear factor kappaB. Oncogene 24: 390–398.

Woodard J, Joshi S, Viollet B, Hay N, Platanias LC (2010) AMPK as a therapeutic target in renal cell carcinoma. Cancer Biol Ther 10: 1168–1177.

Xiang X, Saha AK, Wen R, Ruderman NB Luo Z (2004) AMP-activated protein kinase activators can inhibit the growth of prostate cancer cells by multiple mechanisms. Biochem. Biophys Res Commun 321: 161–167.