Inhibition of adenosine monophosphate-activated protein kinase induces apoptosis in multiple myeloma cells

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In this study, we show that adenosine monophosphate-activated protein kinase (AMPK) is expressed and activated in multiple myeloma cells. The inhibition of AMPK induced growth arrest and reduction of cell viability in the cell viability assay using the water-soluble tetrazolium salt 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1 assay). Induction of apoptosis was determined by annexin-V and propidium iodide staining. The prevention of apoptosis using the pancaspase inhibitor ZVAD-fmk and caspase-3 cleavage upon incubation with the AMPK inhibitor (AMPKI) is shown. Furthermore, incubation of myeloma cells with AMPKI resulted in the downregulation of pAMPK, Mcl-1 and Bcl-x_L. Coincubation of AMPKI and melphalan led to a strong additional increase of apoptosis in myeloma cells. We conclude that AMPKI has a strong antimyeloma activity *in vitro* and represents a new targeted strategy in the treatment of multiple myeloma. *Anti-Cancer Drugs* 18:405–410 © 2007 Lippincott Williams & Wilkins.

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Introduction

Multiple myeloma is still an incurable disease characterized by monoclonal plasma cells that are mainly localized in the bone marrow. In the course of this disease, multiple myeloma cells become resistant to common cytotoxic drugs and patients eventually die of tumour progression. It has been shown that resistance to cytotoxic drugs is associated with changes in protein expression levels of the *bcl-2* family [1,2]. Furthermore, it has been shown that expression levels of Mcl-1 and Bcl-x_L are influenced by cytokines of the bone marrow microenvironment, and therefore provide a molecular target in the therapy of multiple myeloma. Strong and continuous attempts have been made to improve response rates in myeloma patients by novel treatments. Nevertheless, new drugs are urgently needed to encounter this disease.

Adenosine monophosphate-activated protein kinase (AMPK) is a heterotrimeric complex consisting of two regulatory (β and γ) and one catalytic subunit (α) [3]. The kinase is activated by two different mechanisms: First, AMP causes an allosteric activation of AMPK, an activation of AMPK's upstream kinase LKB1 (also known as STK11, serine/threonine kinase 11) and an inhibition of the inhibitory phosphatase. Second, mammalian AMPK is activated not only by a wide variety of cellular stresses that cause energy and ATP depletion, like ischemia, hypoxia, exercise, low glucose levels, osmotic stresses and heat shock, but also by two well-known groups of substances, biguanides and thiazolidinediones [4]. The physiological role is the control of lipid and glucose metabolism [5,6]. Several downstream targets of AMPK are well known. The activation of AMPK leads to an increase of glycolysis, increased fatty acid oxidation, inhibition of 3-hydroxy-3-methlglutaryl-coenzyme A reductase, increased lipolysis and inhibition of cell growth and protein synthesis via the inhibition of mammalian target of rapamycin signalling [4]. The effects of activated AMPK on several tumour types have been studied: activation of AMPK led to antitumour effects like inhibition of proliferation and induction of apoptosis. This has been shown for prostate cancer, chronic lymphatic and myelogenous leukaemia, hepatocellular carcinoma, glioma, astrocytoma, and breast cancer cell lines [7–9].

An inhibition of AMPK has never been studied to encounter tumour growth and to induce apoptosis in tumour cells.

In this study, we demonstrate for the first time that the AMPK inhibitor compound C (AMPKI) induces apoptosis in myeloma cell lines via downregulation of Bcl-x_L and Mcl-1.

Methods

Cells

U266, RPMI8226 and OPM-2 cell lines were obtained from the American Type Culture Collection (Rockville,

Maryland, USA), grown in RPMI-1640 medium (Boehringer, Ingelheim, Germany) containing 10% heat-inactivated fetal calf serum (Boehringer) in a humidified atmosphere (37.5°C; 5% CO_2), and seeded at a concentration of 1×10^5 cells/ml.

Reagents

AMPKI, melphalan, annexin-V binding buffer and propidium iodide (PI) were purchased from Calbiochem (Schwalbach, Germany), and WST-1 from Roche (Penzberg, Germany). ZVAD-fmk was obtained from Biomol (Hamburg, Germany). Polyclonal primary antibodies against AMPK and pAMPK were purchased from Upstate (Hamburg, Germany), antibodies against actin and cleaved caspase-3 were purchased from Cell Signalling (Frankfurt/Main, Germany). Primary antibodies raised against Bim-EL, Bcl-x_L, Mcl-1, Bax and Bcl-2 were obtained from Santa Cruz (Heidelberg, Germany). Secondary antibodies raised against mouse, rabbit and goat epitopes were purchased from Amersham Biosciences (Uppsala, Sweden).

Cell viability assay

For quantification of the cells in suspension, a WST-1 viability assay protocol was used as recommended by the manufacturer (Roche). Absorbance at 440 nm was measured using a microplate enzyme-linked immunosorbent assay reader to detect metabolically intact cells (reference wavelength: 680 nm).

Analysis of apotosis and cell death

Apoptosis was measured by annexin-V and PI staining. Briefly, after two washes with washing buffer (8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4 and 1l H_2O, pH 7.2) cells were resuspended in 400 µl of annexin-V binding buffer. One-hundred microlitres of this cell suspension was incubated with 5 µl of fluorescein isothiocyanate-conjugated annexin-V and 10 µl of 50 µg/ml PI for 15 min at room temperature in the dark. Cells were analysed by flow cytometry (Coulter EPICS XL-MCL; System II; Krefeld, Germany).

Western blot analysis

Western blot analysis was performed as described previously. Cells were lysed with lysis buffer and protein concentrations were determined using the biophotometer. Protein was separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis. After electrophoresis, protein was transferred to nylon membranes (Millipore, Billerica, Massachusetts, USA), blocked in Tween-Tris buffered saline (TBS-T) and 5% nonfat dry milk for 1 h, and subsequently washed and incubated with TBS-T and the primary antibodies for 12 h. After washing with TBS-T, membranes were incubated with a peroxidase-conjugated secondary antibody for 1 h. Signal was detected by chemoluminescene using the ECL detection system (Amersham Biosciences). β-Actin served as internal control for equal loading.

Statistics

Mean values with standard deviations from representative experiments are shown in the figures. Data from the annexin-staining assay were confirmed by two independent experiments. The Mann–Whitney *U*-test was used for statistical analysis of intergroup comparisons. Values of $P < 0.05$ were considered statistically significant.

Results

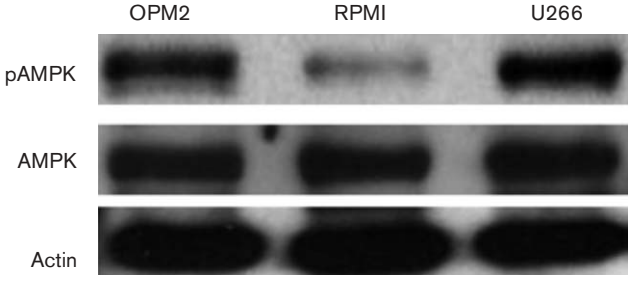
Multiple myeloma cells express adenosine monophosphate-activated protein kinase

Expression of AMPK and pAMPK in the multiple myeloma cell lines OPM2, RPMI8226 and U266 was analysed by Western blot analysis. Our experiments show a band with a molecular mass of 63 kDa representing the AMPK protein. All the three cell lines expressed the protein (Fig. 1). The cell lines OPM2 and U266 expressed higher amounts of activated (phosphorylated) AMPK than the RPMI8226 cell line.

Inhibition of adenosine monophosphate-activated protein kinase reduces cell viability in multiple myeloma cells

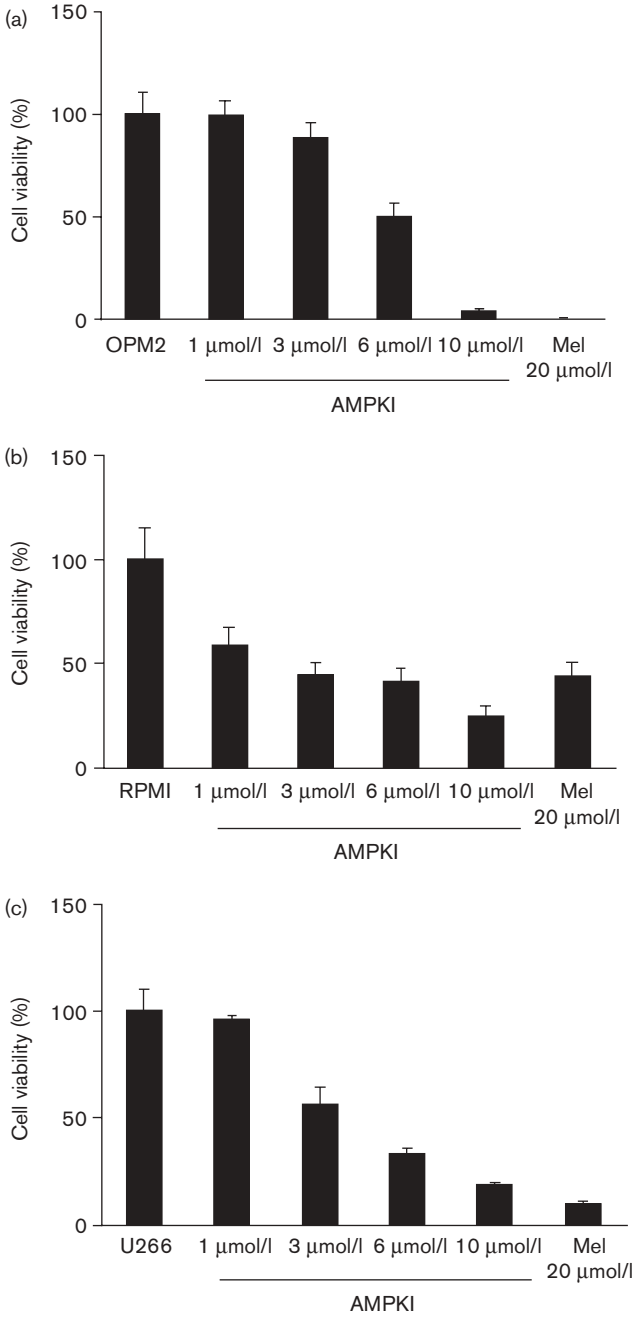
We next examined the cell viability of multiple myeloma cells after incubation with AMPKI. All the three multiple myeloma cell lines were incubated for 48 h with increasing concentrations of AMPKI or 20 µmol/l melphalan. Cell viability was measured with the WST-1 assay. Our experiments show that cell viability of myeloma cells was inhibited after incubation with AMPKI in a dose-dependant manner. Maximum response was seen for OPM2, minimum response for RPMI8226 (Fig. 2a–c).

Fig. 1



Multiple myeloma cells express adenosine monophosphate-activated protein kinase (AMPK). Cell lysates of the myeloma cells lines OPM2, RPMI8226 and U266 were directly subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis, transferred to membranes and blotted with the indicated antibodies.

Fig. 2



Inhibition of adenosine monophosphate-activated protein kinase (AMPK) reduces cell viability in multiple myeloma cells. (a) OPM2, (b) RPMI8226 and (c) U266 myeloma cells were treated with 0, 1, 3, 6 and 10 µmol/l of AMPKI or 20 µmol/l of melphalan for 48 h. Cell viability was determined by the WST-1 assay. Mean values and standard deviations are shown.

Inhibition of adenosine monophosphate-activated protein kinase induces apoptosis in multiple myeloma cells

As the AMPKI reduced cell viability, we next examined whether this is due to induction of apoptosis. OPM2, RPMI8226 and U266 myeloma cells were incubated with increasing concentrations of AMPKI or melphalan for 48 h and apoptosis was analysed by flow cytometry after annexin-V and PI staining. AMPKI induced apoptosis in a dose-dependent manner. The IC_{50} value for the OPM2 cell line lies between 6 and 10 µmol/l, for RPMI cells between 1 and 3 µmol/l, and for U266 cells between 3 and 6 µmol/l (Fig. 3a–c).

Additionally, we incubated U266 and OPM2 myeloma cells with or without 100 µmol/l of the pancaspase inhibitor ZVAD-fmk and 10 µmol/l AMPKI for 48 h, and analysed the cells by flow cytometry using the annexin-V PI assay to re-evaluate induction of apoptosis. Myeloma cells that were coincubated with the pancaspase inhibitor ZVAD-fmk showed reduced levels of annexin-V positivity comparable to untreated cells, indicating that AMPKI leads to caspase activation. ZVAD-fmk showed a greater effect in U266 cells than in OPM2 cells (Fig. 3d–e).

Furthermore, we used Western blot analysis to determine the expression of cleaved caspase-3 in myeloma cells after incubation with AMPKI. To evaluate early downstream signalling events, we reduced the incubation period from 48 to 12 h. U266 cells were incubated with 0, 3 or 6 µmol/l of AMPKI, and then harvested and further processed for Western blot analysis. Inhibition of AMPK with 6 µmol/l for 12 h resulted in a strong cleavage of caspase-3 (Fig. 3f). Interestingly, 3 µmol/l AMPKI do not induce caspase cleavage at this early time point, although this concentration leads to substantial apoptosis after 48 h. This shows that the biological effect of AMPK inhibition is time dependent.

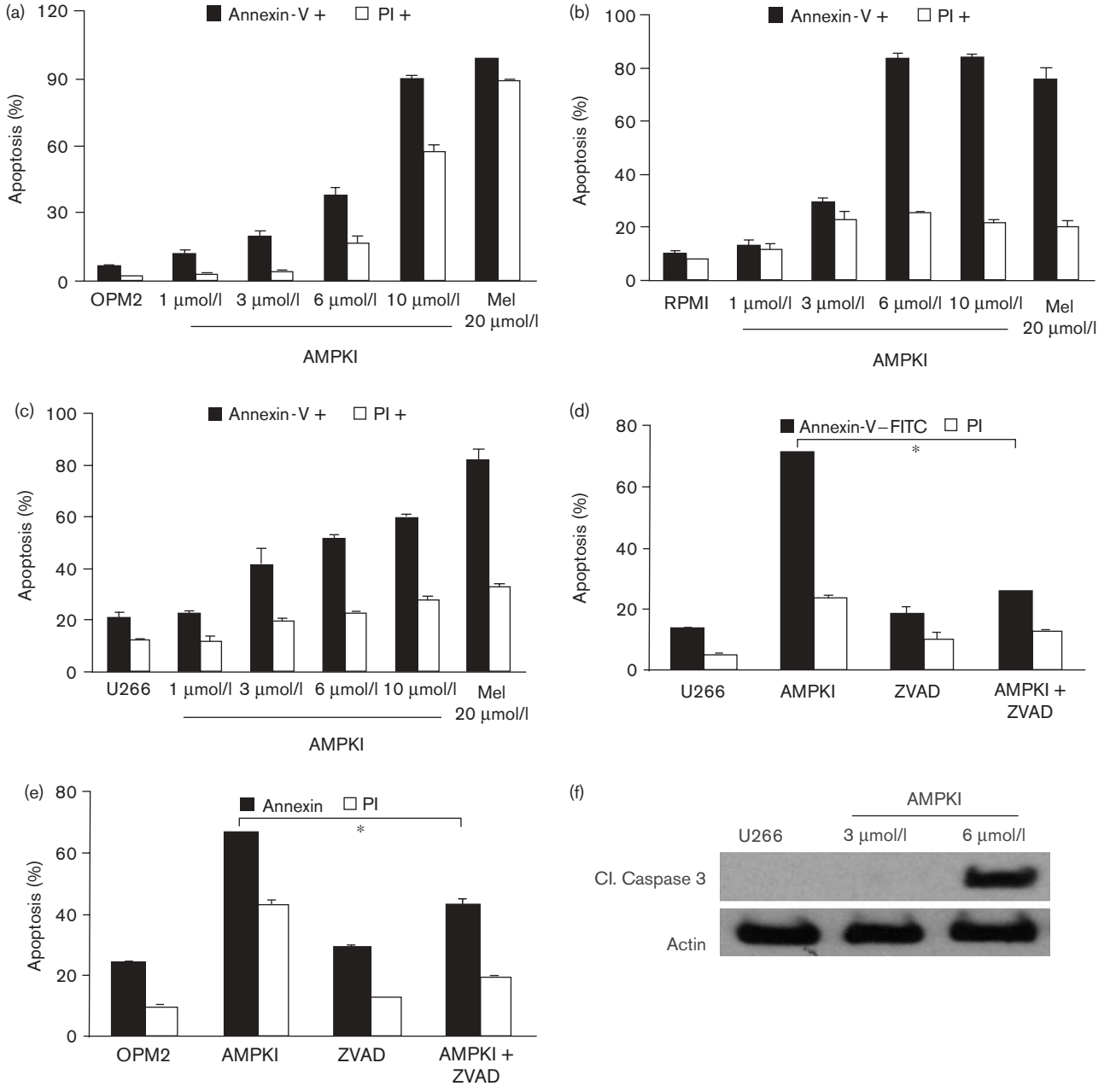
Incubation with adenosine monophosphate-activated protein kinase inhibitor leads to the downregulation of phosphorylated adenosine monophosphate-activated protein kinase, Mcl-1 and Bcl-x_L protein levels

As the incubation of multiple myeloma cells with AMPKI resulted in strong reduction of cell viability and induction of apoptosis, we next analysed the underlying mechanisms.

To show a clear cause–effect relationship, we incubated OPM2 myeloma cells with AMPKI (6 µmol/l) for 2, 4 or 20 h and blotted activated (phosphorylated) AMPK. The used concentration of AMPKI (6 µmol/l) has been shown to induce apoptosis and lead to caspase-3 cleavage in myeloma cells (Figs 2 and 3a–c, f). In our Western blot experiments, incubation of multiple myeloma cells with AMPKI 6 µmol/l resulted in a strong, time-dependent downregulation of pAMPK (Fig. 4a).

Furthermore, we scanned mitochondrial proteins, which are known to have pro- or antiapoptotic characteristics. We therefore incubated U266 multiple myeloma cells

Fig. 3



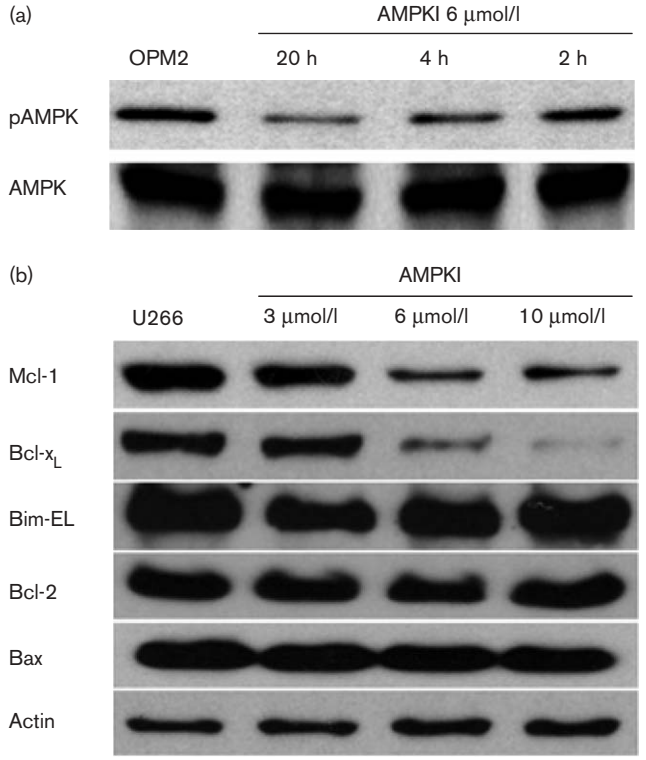
Inhibition of adenosine monophosphate-activated protein kinase (AMPK) induces apoptosis in multiple myeloma cells. (a) OPM2, (b) RPMI8226 and (c) U266 myeloma cells were treated with 0, 1, 3, 6 and 10 μmol/l of AMPKI or 20 μmol/l of melphalan for 48 h. Apoptosis was detected by flow cytometry after staining the cells with annexin-V–fluorescein isothiocyanate (FITC) and propidium iodide (PI). (d) U266 and (e) OPM2 myeloma cells were incubated with 10 μmol/l of AMPKI or 100 μmol/l of ZVAD-fmk or both for 48 h. After harvesting, the number of apoptotic cells was determined by flow cytometry after staining with annexin-V–FITC and PI. (f) U266 myeloma cells were incubated with 0, 3 or 6 μmol/l of AMPKI for 12 h, and protein level of cleaved caspase-3 was determined by Western blotting.

with 0, 3, 6 and 10 μmol/l of AMPKI for 12 h, and then analysed the cells using Western blots. Inhibition or AMPK in myeloma cells resulted in a strong dose-dependent suppression of the antiapoptotic proteins Mcl-1 and Bcl-x_L, whereas the protein levels of Bim-El, Bax and Bcl-2 remained unchanged (Fig. 4b).

Adenosine monophosphate-activated protein kinase inhibitor and melphalan show statistically significant effects on myeloma cells

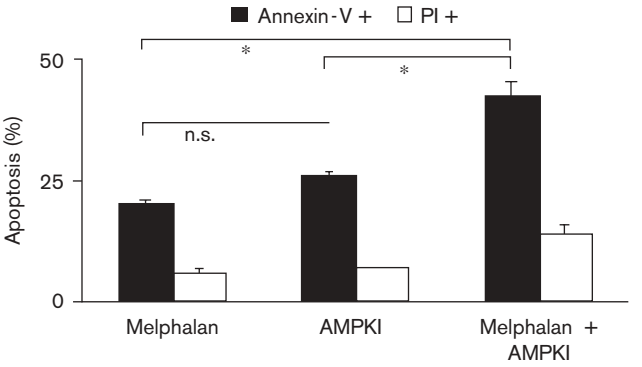
Melphalan is an alkylating agent, which was a gold standard in combination with prednisone, for over 30 years. Only the dose intensification of melphalan –

Fig. 4



Incubation with adenosine monophosphate-activated protein kinase (AMPKI) leads to downregulation of pAMPK, Mcl-1 and Bcl-x_L protein levels. (a) OPM2 myeloma cells were incubated with 6 μmol/l of AMPKI for 2, 4 or 20 h and protein levels of AMPK and pAMPK were determined by Western blotting. Cell lysates were directly subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE), transferred to membranes and blotted with the indicated antibodies. (b) U266 myeloma cells were incubated with none, 3, 6 or 10 μmol/l of AMPKI for 12 h, and protein levels of Bax, Bcl-2, Bim-EL, Mcl-1 and Bcl-x_L were determined by Western blotting. Cell lysates were directly subjected to SDS-PAGE, transferred to membranes and blotted with the indicated antibodies.

Fig. 5



Adenosine monophosphate-activated protein kinase inhibitor (AMPKI) and melphalan show additive effects on multiple myeloma cells. U266 myeloma cells were treated with 3 μmol/l AMPKI, 10 μmol/l melphalan or both for 48 h. Apoptosis was detected by flow cytometry after staining the cells with annexin-V–fluorescein isothiocyanate (FITC) and propidium iodide (PI). To show the drug-induced increase of apoptosis, we subtracted the values of the control (annexin 14.8%; PI 5.6%; not shown) from the values shown above.

enabled by autologous stem cell transplantation – has proven to prolong overall survival [10–12].

Gomez-Bougie *et al.* [13] recently showed that in myeloma cells Mcl-1 and Bim are cleaved, and expression levels are decreased upon incubation with melphalan. Because incubation with AMPKI resulted in a decrease of the Mcl-1 protein, we asked whether AMPKI increases melphalan-induced apoptosis in myeloma cells. We therefore incubated U266 myeloma cells with 3 μmol/l AMPKI or 10 μmol/l melphalan or both and determined apoptosis after 48 h. As we expected, coincubation of melphalan with AMPKI nearly doubled apoptosis rates in myeloma cells (Fig. 5).

Discussion

AMPK is a key enzyme for the control of lipid and glucose metabolism. It has been shown that the activation leads to a decrease of proliferation of tumour cells. Nothing is known about the effect on tumour cells when AMPK is inhibited using AMPKI. This substance selectively inhibits AMPK and has no influence on Syk-kinase, protein kinase Cθ, protein kinase A or janus kinase-3 [14]. So far, the AMPKI inhibitor Compound C has been solely used to antagonize the allosteric activation of AMPK with 5-Aminoimidazole-4-carboxamide-1-b-riboside (AICAr), a widely used AMPK activator.

To our own surprise, AMPKI strongly reduced cell viability of multiple myeloma cells. Further experiments revealed that AMPKI induced a significant increase in apoptosis. Additionally, apoptosis was reversed when the cells were coincubated with the pancaspase inhibitor ZVAD-fmk. Furthermore, we were able to show caspase-3 cleavage in U266 cells at a concentration of 6 μmol/l, surprisingly not at 3 μmol/l, which has been shown to induce annexin-V–fluorescein isothiocyanate binding after 48 h incubation. This can be explained by the decreased incubation period (12 h) used in the Western blot experiments. It is still unclear why the AMPKI compound C induces apoptosis in myeloma cells, whereas in other tumour types the activation of AMPK induces inhibition of proliferation. One can argue that like plasma cells – the healthy counterparts – multiple myeloma cells are terminally differentiated resting cells with low proliferative capacity and that therefore the proliferation is no more under the control of AMPK. Further comparative studies are, however, needed to elucidate the differences in the pathophysiology of proliferation control.

We scanned U266 myeloma cells for changes in the protein expression levels of AMPK, pAMPK and of members of the *bcl-2* family. Myeloma cell incubation with AMPKI resulted in a downregulation of activated AMPK. Furthermore, it led to a downregulation of the anti-apoptotic proteins Bcl-x_L as well as Mcl-1. Bcl-x_L and Mcl-1 are both well-known proteins in the regulation of myeloma cell apoptosis [15–17]. Compared with nonmalignant plasma cells, Mcl-1 is overexpressed in multiple myeloma cells and the level of Mcl-1 expression is related to disease severity [14]. Both an interleukin-6-dependent and -independent regulation of the Mcl-1 protein has been discussed in the past [18,19]. Bcl-x_L is also known to be upregulated upon myeloma cell stimulation with interleukin-6 and therefore contributes to myeloma cell survival [20].

Gomez-Bougie *et al.* [13] recently showed that in myeloma cells Mcl-1 and Bim are reduced upon incubation with melphalan. We therefore asked whether the combination of these drugs enhances rates of apoptosis and incubated myeloma cells with both drugs. As we expected, induction of apoptosis in myeloma cells was strongly increased when both drugs were added simultaneously.

We conclude that AMPKI has a strong antimyeloma activity *in vitro* and represents a new targeted strategy in the treatment of multiple myeloma.

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