Myeloma cell growth inhibition is augmented by synchronous inhibition of the insulin-like growth factor-1 receptor by NVP-AEW541 and inhibition of mammalian target of rapamycin by Rad001

Philipp Baumann, Hilke Hagemeier, Sonja Mandl-Weber, Daniel Franke and Ralf Schmidmaier

Multiple myeloma is still incurable. Myeloma cells become resistant to common drugs and patients eventually die of tumour progression. Therefore, new targets and drugs are needed immediately. NVP-AEW541 is a new, orally bioavailable small molecule inhibitor of the insulin-like growth factor-1 receptor (IGF-1R). Here, we show that NVP-AEW541 inhibits cell growth in myeloma cells at low concentrations in a time-dependent and a dose-dependent manner. Further experiments using the annexin-V–fluorescein isothiocyanate/propidium iodide assay revealed induction of apoptosis in common myeloma cell lines, but not in peripheral blood mononuclear cell from healthy donors. Stimulation of myeloma cells with IGF-1 led to a vast increase of cell growth and this was blocked by low doses of NVP-AEW541. Stimulation of myeloma cells with conditioned medium obtained from a 48-h-old HS-5 stromal cell culture was only partly blocked by NVP-AEW541. Western blotting experiments revealed that NVP-AEW541 decreased the phosphorylation status of P70S6 kinase and 4E-BP-1 but not of mammalian target of rapamycin (mTOR). Combined inhibition of IGF-1R and mTOR using the novel mTOR inhibitor Rad001 led to additive/synergistic increase of cell growth inhibition in multiple myeloma cells, which was accompanied by a stronger dephosphorylation of P70S6 kinase and 4E-BP-1. Taken together, we show that the combined inhibition of IGF-1R and mTOR by combining NVP-AEW541 and Rad001 is highly effective in multiple myeloma and might represent a potential new treatment strategy. *Anti-Cancer Drugs* 20:259–266 © 2009 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Keywords: Akt, mammalian target of rapamycin, multiple myeloma, NVP-AEW541, phosphatidylinositol 3 kinase, Rad001

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Introduction

Multiple myeloma (MM) is characterized by plasma cells that accumulate in the bone marrow and produce a monoclonal immunoglobulin. Clinical manifestations include lytic bone lesions, anaemia, hypercalcaemia, renal failure and others. MM remains incurable, accounting for approximately *2%* of all neoplastic diseases [1]. In the last decade, treatment of MM has changed rapidly. The median survival of patients treated with conventional cytotoxic drugs is approximately 3 years only. The therapy with high-dose regimes accompanied by autologous stem cell support, as well as the introduction of new substances, such as bortezomib, thalidomide and lenalidomide, have prolonged median overall survival. Despite these advances, MM cells become resistant to cytotoxic drugs and patients eventually die of tumour progression [2–7].

In MM, the malignant plasma cell clone is found in the bone marrow microenvironment, which has been shown to support survival, drug resistance and proliferation of myeloma cells. This is meant to be mediated by both cellular adhesion [8,9] as well as growth factors such as insulin-like growth factor-1 (IGF-1) and vascular endothelial growth factor, and cytokines such as interleukin (IL)-6, IL-4, stromal-derived factor-1 and others [10,11]. IGF-1 plays a central role in the stimulation of cell growth and inhibition of apoptosis [12,13]. Binding of IGF-1 to its receptor results in the autophosphorylation of tyrosine residues of the intracellular domain of the receptor, which in turn leads to the activation of signalling pathways further downstream, the phosphatidylinositol 3 kinase (PI3k) and Akt pathways and the Ras/Raf/mitogen-activated protein (MAP) kinase signalling pathway [14–16]. Both signalling pathways are known to induce cellular growth, proliferation, drug resistance and the spreading of malignant cells. Therefore, inhibition of IGF-1 signalling is a molecular target in the treatment of MM.

NVP-AEW541 belongs to the pyrrol [2,3-d] pyrimidine class and has shown to be a new, small molecule, selective

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IGF-1 receptor (IGF-1R) inhibitor [17]. In this study, we show that this IGF-1R inhibitor is a potent inhibitor of myeloma cell growth and induces apoptosis in MM cell lines. Furthermore, we show that it inhibits the Akt/mammalian target of rapamycin (mTOR)/P70S6 kinase (P70S6k)/4E-BP-1 pathway and increases cytotoxicity triggered by the new mTOR inhibitor Rad001 (everolimus). Taken together, NVP-AEW541 might represent a promising drug in the therapy of MM.

Methods

Cells

U266, NCI-H929 and OPM-2 cell lines and the human stromal cell line HS-5 were obtained from the American Type Culture Collection (Rockville, USA), grown in RPMI 1640 medium (Boehringer, Ingelheim, Germany) containing 10% heat-inactivated foetal calf serum (Boehringer) in a humidified atmosphere (37.5°C; 5% CO_2), and seeded at a concentration of 1×10^5 cells/ml. Cells were regularly tested for mycoplasma and were free of this contamination.

Reagents

Rad001 (everolimus) and propidium iodide were purchased from Calbiochem (Schwalbach, Germany) and WST-1 from Roche (Penzberg, Germany). NVP-AEW541 was kindly provided by Novartis Company (Basel, Switzerland). Polyclonal primary antibodies against actin were obtained from Santa Cruz (Heidelberg, Germany) and phosphorylated (p)-P70S6k (Thr421/Ser424), p-mTOR (Ser2448), p-4E-BP-1 (Thr70), P70S6k, mTOR and 4E-BP-1 from Cell Signaling (Frankfurt/Main, Germany). Secondary antibodies raised against mouse, goat or rabbit epitopes were purchased from Amersham Biosciences (Uppsala, Sweden).

Cell growth assay

For quantification of the cells in suspension, a WST-1 growth inhibition assay protocol was used as recommended by the manufacturer (Roche). After an incubation period of 48 h the WST-1 agent was added to the myeloma cell culture. After another hour, absorbance at 440 nm was measured using a microplate enzyme-linked immunosorbent assay reader (Berthold Technologies, Bad Wildbad, Germany) (reference wavelength: 680 nm).

Analysis of apoptosis and cell death

Cells were stained with propidium iodide and annexin-V–fluorescein isothiocyanate. 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 1 l H_2O, pH 7.2), cells were resuspended in 400 µl of Dulbecco's PBS. One hundred microliters of this cell suspension were incubated with 10 µl of 50 µg/ml propidium iodide and 5 µl of annexin-V–fluorescein isothiocyanate for 15 min at room temperature in the dark. Cells were analysed by flow cytometry (Coulter EPICS XL-MCL; System II, Beckman Coulter, 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 SDS–polyacrylamide gel electrophoresis. After electrophoresis, protein was transferred to nylon membranes (Millipore, Massachusetts, USA), blocked in Tris-buffered saline with Tween 20 (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. Signals were detected by chemoluminescence using the ECL detection system (Amersham Biosciences). Beta-actin served as internal control for equal loading.

Statistics

Mean values with standard deviations from representative experiments are shown in the figures. Data were confirmed by at least two independent experiments. The Wilcoxon's test was used to compare different groups. Values of P less than 0.05 were considered statistically significant.

Results

NVP-AEW541 inhibits cell growth in multiple myeloma cells

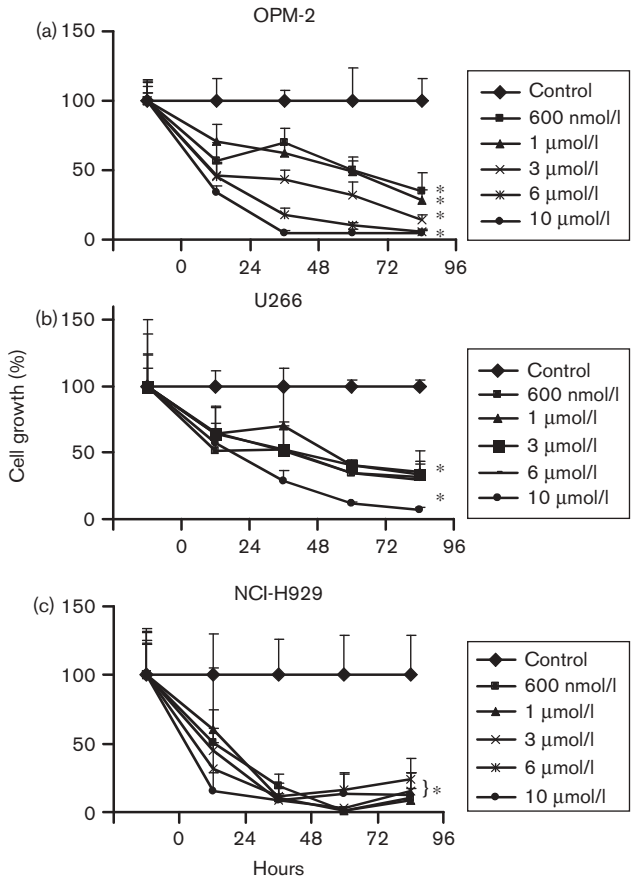
First, the effect of NVP-AEW541 on myeloma cell growth was determined by the WST-1 assay. In this assay, the tetrazolium salt (WST-1) is changed to formazan by the succinate–tetrazolium reductase, which is found only in the mitochondrial respiratory chain. The quantity of formazan dye is therefore directly related to the number of metabolically active cells and was quantified by an enzyme-linked immunosorbent assay reader.

OPM-2, NCI-H929 and U266 myeloma cells were incubated with increasing concentrations of NVP-AEW541 for 96 h; the medium was not changed during the course of the experiment. Cell growth was determined every 24 h. As shown in Fig. 1, myeloma cell growth was inhibited in a time-dependent and a dose-dependent manner. All three cell lines showed good response rates. After 24-h incubation, the half maximal inhibitory concentration values were found to be between 600 nmol/l and 1 µmol/l.

Inhibition of insulin-like growth factor-1 receptor induces apoptosis in myeloma cell lines

To analyse whether inhibition of IGF-1R may induce apoptosis in myeloma cells, we performed the annexin-V/propidium iodide assay. Cells of all three cell lines were incubated with increasing concentrations of NVP-AEW541 for 72 h. After the incubation period, cells were harvested and further processed for flow cytometry

Fig. 1



NVP-AEW541 inhibits cell growth in multiple myeloma (MM) cells. (a) OPM-2, (b) U266 and (c) NCI-H929 MM cell lines were incubated with none, 600 nmol/l, 1, 3, 6 or 10 µmol/l of NVP-AEW541 for 24, 48, 72 and 96 h and cell growth was determined by the WST-1 assay. Mean values and standard deviations are shown. The figures show results from two independent experiments. *$P<0.05$ versus control.

analysis. As shown in Fig. 2a–c, significant induction of apoptosis occurred in all three cell lines. In contrast to U266 cells, OPM-2, NCI-H929 myeloma cells showed annexin-V positivity after incubation with low doses of NVP-AEW541 (OPM-2: 49% at 1 µmol/l; NCI-H929: 59% at 1 µmol/l and U266: 18% at 6 µmol/l). In contrast to MM cell lines, induction of apoptosis did not occur in lymphocytes donated by healthy volunteers (Fig. 2d). In addition, we performed a time course assay and incubated NCI-H929 myeloma cells with 1 µmol/l of NVP-AEW541 for up to 72 h and determined apoptosis every 24 h. We clearly can show that induction of apoptosis occurs in a time-dependent manner (20% annexin-V-positive after 48 h and 50% annexin-V-positive after 72 h, Fig. 2e).

NVP-AEW541 inhibits insulin-like growth factor-1-induced cell growth

In MM, plasma cells accumulate in the bone marrow. Several studies have shown that cytokines and growth factors such as IGF-1 stimulate plasma cell growth and induce proliferation and cell survival in plasma cells by activating the PI3k/Akt pathway. To show that the inhibition of IGF-1R inhibits the stimulatory effect of IGF-1, we performed another cell growth assay using the WST-1 assay. NCI-H929 and U266 myeloma cells were incubated with increasing concentrations of NVP-AEW541 and 200 ng/ml IGF-1 in RPMI 1640 without foetal calf serum. Myeloma cells responded strongly to the stimulation with IGF-1 (NCI-H929: + 350%; U266: + 70%). Upon inhibition with NVP-AEW541, cell growth was strongly inhibited and the stimulatory effect on cell growth exerted by IGF-1 was totally abrogated (Fig. 3a and b).

In further experiments, we examined whether NVP-AEW541 also blocked the stimulatory effect of conditioned medium obtained from a 48 h HS-5 stromal cell culture. MM cells were incubated with increasing concentrations of NVP-AEW541 and stimulated with or without conditioned medium containing 50% HS-5 stromal cell medium. Again, MM cell responded to the stimulation with conditioned medium (NCI-H929: + 11%; U266: + 350%). The simultaneous inhibition of IGF-1R only moderately inhibited increased cell growth induced by conditioned medium (Fig. 3c and d), suggesting that NVP-AEW541 selectively inhibits IGF-1R and not other tyrosine kinase receptors.

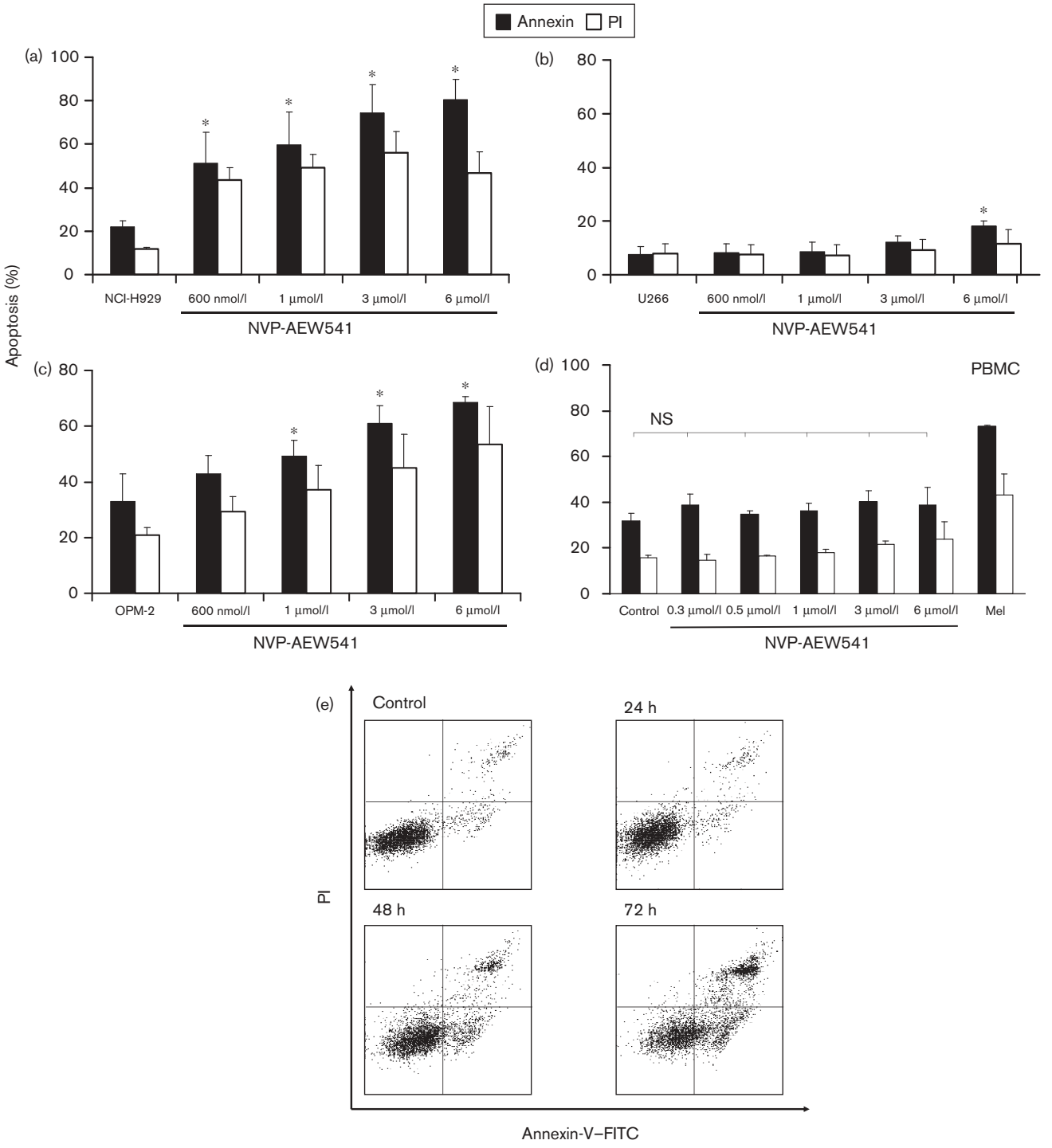
NVP-AEW541 inhibits P70S6 kinase and 4E-BP-1

It has been shown before that myeloma cell growth is closely related to the activation of the PI3k/Akt pathway [18–21]. Maiso *et al.* [22] have shown that NVP-AEW541 strongly inhibits the phosphorylation of IGF-1R and Akt. As p-P70S6k and p-4E-BP-1 strongly contribute to drug resistance, inhibition of apoptosis and cell growth, we analysed whether NVP-AEW541 also leads to the inhibition of mTOR signalling and signalling beyond mTOR, P70S6k and 4E-BP-1. We therefore performed western blotting experiments to show the inhibitory effects of this substance on the three signalling molecules mTOR, P70S6k and 4E-BP-1 in MM cells. We therefore cultured NCI-H929 and OPM-2 myeloma cells for 2 h with or without 200 ng/ml IGF-1 and 1 µmol/l NVP-AEW541. IGF-1 alone induced a strong increase of phosphorylation of P70S6k and 4E-BP-1 and only a moderate increase of phosphorylation of mTOR. NVP-AEW541 alone inhibited phosphorylation of P70S6k and 4E-BP-1, but not mTOR. When IGF-1 and NVP-AEW541 were both added to the cell culture, NVP-AEW541 overcame phosphorylation of P70S6k and 4E-BP-1, but as before, the phosphorylation of mTOR was not or was only marginally altered by NVP-AEW541 (Fig. 4a and b).

NVP-AEW541 exerts synergism with the new mammalian target of rapamycin inhibitor Rad001

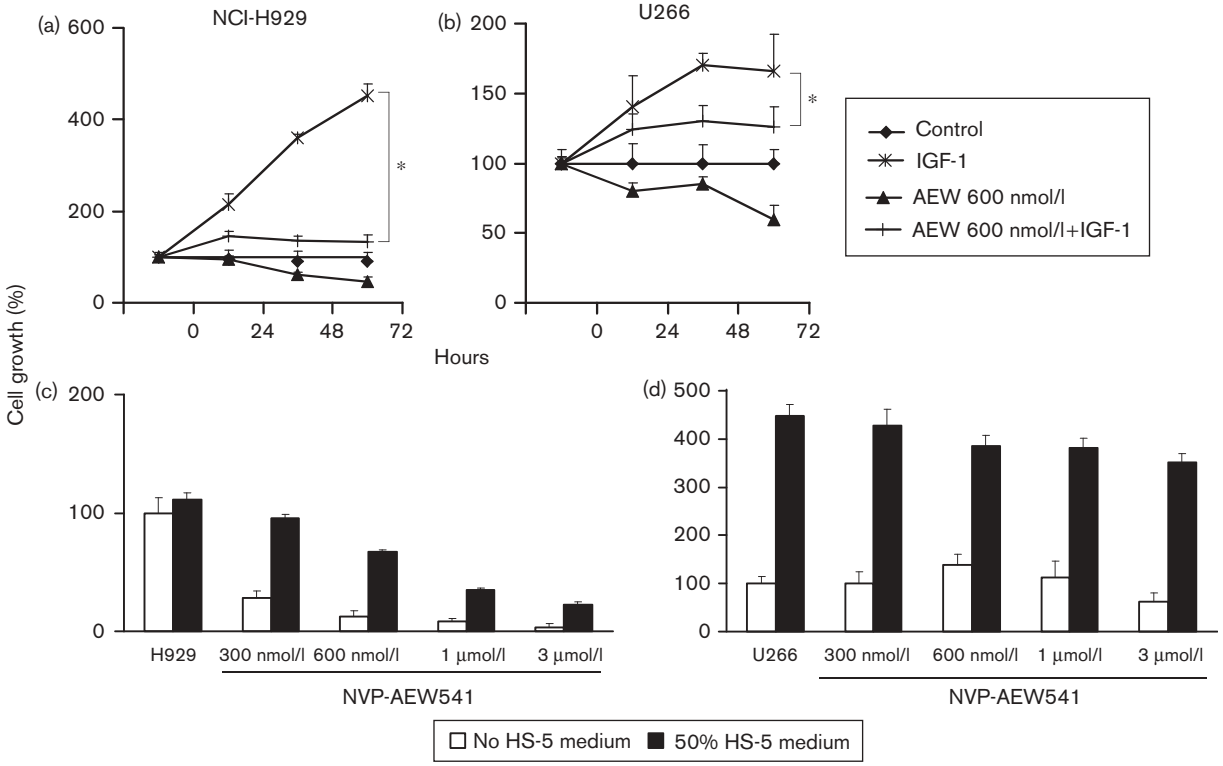
As western blotting analysis showed that the phosphorylation status of mTOR is not altered upon stimulation with

Fig. 2



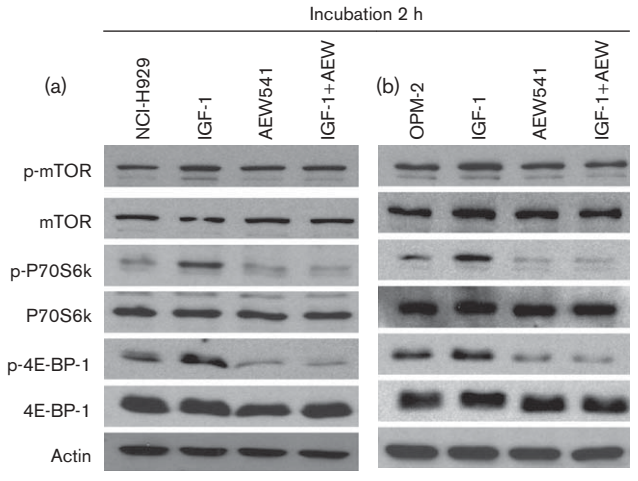
NVP-AEW541 induces apoptosis in common multiple myeloma cell lines but not in peripheral blood mononuclear cells (PBMCs) from healthy donors. (a) NCI-H929, (b) U266 and (c) OPM-2 were incubated with 600 nmol/l, 1, 3 and 6 μmol/l of NVP-AEW541 for 48 h and induction of apoptosis was determined after annexin-V–fluorescein isothiocyanate (FITC)/propidium iodide (PI) staining by flow cytometry. (d) PBMCs obtained from two healthy donors were incubated with 0.3, 0.5, 1, 3 and 6 μmol/l of NVP-AEW541 or 10 μmol/l melphalan (Mel) for 72 h and induction of apoptosis was determined after annexin-V–FITC/PI staining by flow cytometry. The figures show results from two independent experiments. *$P < 0.05$ versus control. NS indicates not significant. (e) NCI-H929 myeloma cells were incubated with 1 μmol/l of NVP-AEW541 for up to 72 h and induction of apoptosis was determined every 24 h by the annexin/PI assay.

Fig. 3



NVP-AEW541 inhibits insulin-like growth factor-1 (IGF-1)-induced increase of myeloma cell growth. (a) NCI-H929 and (b) U266 myeloma cells were incubated with/without 200 ng/ml of IGF-1 for 48 h. Cell growth was inhibited by adding 600 nmol/l of NVP-AEW541 to the culture. Cell growth was determined by using the WST-1 assay. The figures show results from two independent experiments. *$P<0.05$ versus control. NS indicates not significant. (c) NCI-H929 and (d) U266 myeloma cells were incubated with conditioned medium obtained from a 2-day-old HS-5 stromal cell culture for 48 h. Cell growth was determined by using the WST-1 assay. The figures show results from two independent experiments.

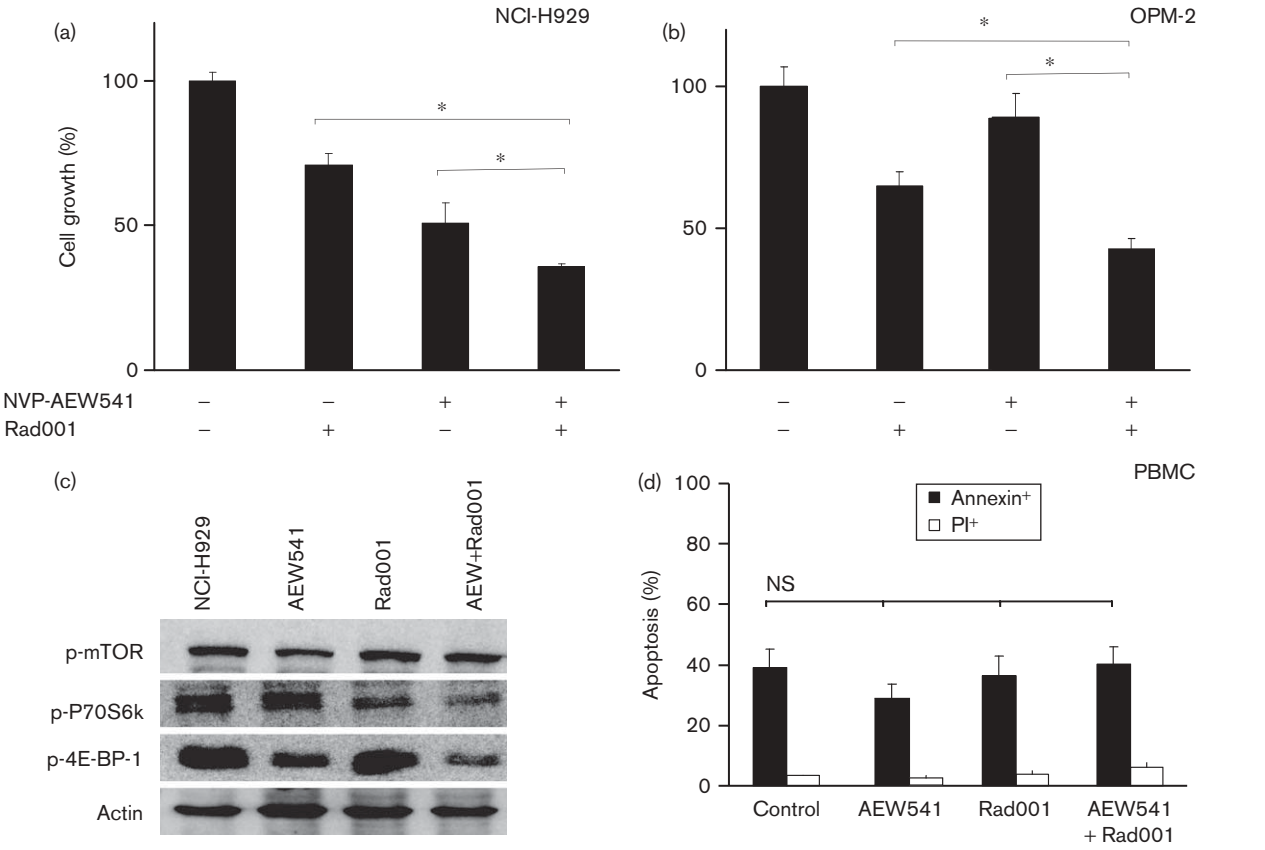
Fig. 4



Incubation of myeloma cells with NVP-AEW541 leads to decreased phosphorylation of mediators of the mammalian target of rapamycin (mTOR) pathway. (a) NCI-H929 and (b) OPM-2 myeloma cells were incubated with 200 ng/ml insulin-like growth factor-1 (IGF-1) and/or 1 μmol/l of NVP-AEW541 for 2 h. After the incubation period cells were lysed and directly subjected to SDS–polyacrylamide gel electrophoresis, transferred to membranes and blotted with indicated antibodies.

NVP-AEW541, we examined whether the combination of NVP-AEW541 and the new mTOR inhibitor Rad001 (everolimus) additionally inhibits cell growth in MM cells. NCI-H929 and OPM-2 cells were treated with NVP-AEW541 600 nmol/l and with or without 100 nmol/l Rad001 (everolimus) and were cultured for 48 h. Cell growth was determined by the WST-1 assay. Figure 5a and b shows that when NVP-AEW541 was combined with Rad001 (everolimus), additive as well as synergistic effects were observed concerning cell growth. Increase of cell growth inhibition was seen to a larger extent in NCI-H929 MM cells than in OPM-2 cells. Analysis using the Calcusyn software (Biosoft, Cambridge, UK) revealed that the combination of NVP-AEW541 with Rad001 leads to additive and synergistic effects in MM cells (Table 1). Furthermore, we analysed whether the combined inhibition of IGF-1R and mTOR leads to an increased inhibition of P70S6k and 4E-BP-1, we performed western blotting experiments. Incubation of NCI-H929 MM cells with 1 μmol/l NVP-AEW541 and 100 nmol/l Rad001 revealed that the combination led to a stronger inhibition of phosphorylation of P70S6k and 4E-BP-1 than each substance alone (Fig. 5c). Furthermore, we examined whether the combination of NVP-AEW541

Fig. 5



NVP-AEW541 exerts synergistic activity on myeloma cells when it is combined with the new mammalian target of rapamycin inhibitor Rad001. (a) NCI-H929 and (b) OPM-2 multiple myeloma cell lines were incubated with 600 nmol/l of NVP-AEW541 or 100 nmol/l of Rad001 or the combination of both drugs for 48 h. Cell growth was determined by the WST-1 assay. Mean values and standard deviations are shown. The figures show results from two independent experiments. *$P<0.05$ versus control. NS indicates not significant. (c) NCI-H929 myeloma cells were incubated with 600 nmol/l of NVP-AEW541 or 100 nmol/l of Rad001 or the combination of both drugs for 2 h. After the incubation period cells were lysed and directly subjected to SDS–polyacrylamide gel electrophoresis, transferred to membranes and blotted with indicated antibodies. (d) Peripheral blood mononuclear cells (PBMCs) from two healthy donors were incubated with 600 nmol/l of NVP-AEW541 or 100 nmol/l of Rad001 or both for 48 h and induction of apoptosis was determined after the incubation period by the annexin/propidium iodide (PI) assay. NS indicates not significant.

Table 1 NCI-H929 and OPM-2 multiple myeloma cells were incubated with NVP-AEW541 (300 and 600 nmol/l)

NVP-AEW541	Rad001	FA	CI
NCI-H929			
300	100	0.403283	0.585
300	500	0.403283	0.597
600	100	0.220552	0.668
600	500	0.230447	0.693
OPM-2			
300	100	0.649662	1.090
300	500	0.63	3.590
600	100	0.428829	0.170
600	500	0.466216	0.281

After 1 h, Rad001 (100 or 500 nmol/l) was added and cells were incubated for 48 h. Then, cell growth was determined by the WST-1 assay. Data obtained from these experiments were analysed using the Calcusyn software to show additive/synergistic effects. Table 1 shows combination index (CI) values obtained from experiments using the OPM-2 and NCI-H929 cell lines. CI: 1.1–0.9 indicates additive effects, whereas CI<0.9 indicates synergistic effects.
FA, fraction affected.

and Rad001 leads to an induction of apoptosis in peripheral blood mononuclear cells of healthy donors and performed an annexin/propidium iodide apoptosis assay. We can show that even the combination of both drugs does not induce apoptosis in peripheral blood mononuclear cells (Fig. 5d).

Discussion

IGF-1 is an important survival factor for MM cells and cell lines [23]. Bataille *et al.* [24] have shown that expression of the IGF-1R CD221 on MM cells is a negative prognostic factor in MM patients. In addition, earlier studies have shown that the levels of IGF-1 are not necessarily increased in myeloma patients but increased levels of IGF-1 are associated with poor prognosis [25].

Mechanistically, IGF-1 binds to the IGF-1R and activates the PI3k/Akt pathway and the MAP kinase pathway in myeloma cells without activation of the Janus kinase/signal transducer and activator of transcription signalling pathway. Activation of the Akt signalling cascade leads to well-known cellular events that lead to increased proliferation, myeloma cell migration, cellular adhesion, cell growth and resistance to cytotoxic drugs [26,27].

We reevaluated the effect of NVP-AEW541 in MM cells. NVP-AEW541 strongly inhibited cell growth in the low micromolar range. Furthermore, it induced substantial apoptosis in the NCI-H929 and OPM-2 cell lines, whereas induction of apoptosis in the U266 cell line was observed only at higher doses. These results stand in line with the results of Maiso *et al.* [22] who have obtained comparable results.

In MM, the malignant plasma cell clone is localized in the bone marrow microenvironment, which has been shown to endorse survival and cell proliferation of myeloma cells. Cytokines and growth factors play a crucial role in this interaction. Mediators of protective effects are growth factors such as IGF-1 and vascular endothelial growth factor, and cytokines such as IL-6, IL-4 and others. Therefore, we investigated the effect of NVP-AEW541 myeloma cells cultured with IGF-1. As expected, we saw that NVP-AEW541 abrogates the IGF-1-induced cell growth of MM cells. Furthermore, we investigated the effect of conditioned medium obtained from HS-5 bone marrow stromal cells in MM cells treated with NVP-AEW541. In contrast to Maiso *et al.* [22], we saw only a modest effect on MM cells stimulated with conditioned medium. This result is not astonishing, as bone marrow stromal cells not only synthesize IGF-1, but also a large amount of other growth factors and cytokines that can confer to survival and cell growth are synthesized [28].

Mechanistic studies have shown that NVP-AEW541 modulates cell cycle regulators, mitochondrial proapoptotic and antiapoptotic proteins, MAP kinase and protein kinase B (Akt). As the PI3k/Akt/mTOR pathway is crucial for cell growth and survival, we analysed whether inhibition of this pathway using NVP-AEW541 also modulates the expression of cell signalling proteins downstream of Akt. In MM cells, Akt activates mTOR, a protein related to cell growth. Mammalian TOR in turn activates P70S6k and 4E-BP-1, which both increase protein translation and cell growth [29,30], cell cycle progression [31] and inhibition of apoptosis [32,33]. Our western blotting experiments revealed that NVP-AEW541 leads to a strong inhibition of phosphorylation of P70S6k and 4E-BP-1.

Surprisingly, and in contrast to effects shown on P70S6k and 4E-BP-1, we detected a moderate or even no dephosphorylation of mTOR upon incubation with NVP-AEW541. To further increase the inhibition of cell growth in MM cells, we coincubated MM cells with NVP-AEW541 and Rad001, a second-generation mTOR inhibitor, which is already in clinical use in MM. WST-1 cell growth assays proved that coincubation of both substances leads to additive and synergistic effects in MM cells. Western blotting experiments confirmed that the augmentation of cell growth inhibition is because of a further decrease of the phosphorylation status of P70S6k and 4E-BP-1.

Taken together, we show that the highly selective IGF-1R inhibitor NVP-AEW541 inhibits cell growth and induces apoptosis in MM cells. Furthermore, we show that incubation of MM cells with NVP-AEW541 leads to a strong inactivation of P70S6k and 4E-BP-1. Dual inhibition within the same pathway, using the new mTOR inhibitor Rad001, further increased MM cell growth inhibition. Our findings impact on the development of anti-IGF-1 treatment options.

Acknowledgements

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