Synergistic antimyeloma effects of zoledronate and simvastatin

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Despite advances in the treatment of multiple myeloma, it remains an incurable disease because of primary and secondary drug resistance. Mevalonate pathway inhibitors like bisphosphonates and statins have antimyeloma activity *in vitro* at very high concentrations, which may probably not be reached *in vivo*. NCI-H929, OPM-2, U266 and RPMI-8226 myeloma cell lines were treated in the presence or absence of bone marrow stromal cells with simvastatin or zoledronate in combination with classical antimyeloma drugs like melphalan or bortezomib. Zoledronate did not show substantial antimyeloma activity at low and intermediate concentrations, whereas simvastatin potently induced apoptosis in myeloma cells without signs of primary, cell-adhesion-mediated drug resistance. Furthermore, sequential blockage of the mevalonate pathway by zoledronate and simvastatin demonstrated synergistic induction of apoptosis and reversal of cell-adhesion-mediated drug resistance. Our data provide a rationale for combining zoledronate and simvastatin with classical antimyeloma drugs.
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Introduction

Multiple myeloma is an incurable hematological disease with the accumulation of malignant plasma cells in the bone marrow. High-dose chemotherapy and the introduction of new drugs have significantly improved the prognosis over the last decade. Nevertheless, only about one-third of patients respond to monotherapy of the new drugs and virtually all patients eventually relapse after high-dose chemotherapy. Therefore, further improvements of the current treatments are needed and may be achieved either by new drugs or by optimized combination of available compounds.

Bisphosphonates are synthetic analogues of pyrophosphate and have been shown to be specific inhibitors of osteoclastic activity. As several phase III trials have demonstrated that intravenous monthly infusions of either pamidronate or zoledronate reduce the skeletal complications among multiple myeloma patients, bisphosphonates are now a mainstay of myeloma therapy (for a review, see [1]). In addition to the antiresorptive effects, bisphosphonates have shown antimyeloma effects *in vitro*, in murine models, as well as in clinical studies [2] and clinical observations [3]. The available published data regarding antimyeloma activity of both in-vitro and in-vivo studies, however, are in part contradictory and the effects are often only minimal. To achieve clinically relevant antimyeloma effects, dose intensification of pamidronate was performed, but failed due to increased side effects. Current data regarding zoledronate, however, suggest that doses of up to 12 mg instead of the standard 4 mg can be administered safely. Further studies are needed to evaluate a possible antimyeloma activity. Alternatively, we hypothesized that the combination with other mevalonate pathway inhibitors may increase antimyeloma activity of these compounds. Bisphosphonates are known inhibitors of the mevalonate pathway. First-generation bisphosphonates (clodronate, etidronate, tiludronate) inhibit the conversion of mevalonate to isopentenyl-pyrophosphate, second-generation bisphosphonates (pamidronate, alendronate) are inhibitors of the dimethylallyl-*trans*-transferase and third-generation bisphosphonates (ibandronate, risedronate, zoledronate) additionally inhibit the geranyl-*trans*-transferase.

Statins, like simvastatin, inhibit the 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase, which catalyzes the conversion of HMG-CoA to mevalonate. Interestingly, in multiple myeloma models statins induce apoptosis [4–11], inhibit proliferation [5,9,11,12], overcome primary [13] and secondary [9,11] drug resistance, and synergize with cytotoxic drugs [5,8,9,13]. Clinical trials to investigate the *in vivo* antimyeloma activity of statins and the feasibility of high-dose statins in combination with cytotoxic drugs are ongoing. Additionally, preclinical studies regarding the combination of

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statins and bisphosphonates in multiple myeloma are obviously needed.

The data presented clearly show synergistic effects of simvastatin and zoledronate in terms of induction of apoptosis and overcoming primary bortezomib resistance. The combination of statins, bisphosphonates and approved cytotoxic antimyeloma compounds may further improve response rates and survival in clinical trials.

Materials and methods

Cells

NCI-H929, U266, RPMI-8226, OPM-2 and HS-5 cell lines were obtained from the American Type Culture Collection (Rockville, Maryland, USA), grown in RPMI 1640 medium (Boehringer, Ingelheim, Germany) containing 20% 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. After informed consent, mononuclear cells from bone marrow aspirates were grown in plastic flasks to a confluent, adherent monolayer. The local ethical committee of the University of Munich approved the study.

Reagents

Simvastatin, clodronate, zoledronate, and propidium iodide (PI) were purchased from Sigma-Aldrich (Seelze, Germany), bortezomib from Ortho Biotech (Janssen-Cilag, Neuss, Germany), pamidronate from Novartis Pharma (Nürnberg, Germany), CD138–phycoerythrin and annexin V–fluorescein isothiocyanate (FITC) from Pharmingen (BD Biosciences, Heidelberg, Germany), CD38–FITC from von Immunotech (Beckman Coulter, Krefeld, Germany), and water-soluble tetrazolium salt (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) (WST-1) from Roche (Penzberg, Germany).

Analysis of apoptosis and cell death

Cells were stained with fluorescein-conjugated annexin V and PI. 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 binding buffer (10 mmol/l *N*-2-hydroxyl piperazine-*N*′-2-ehane sulfonic acid/NaOH, pH 7.4, 140 mmol/l NaCl, 2.5 mmol/l $CaCl_2$). One hundred microliters of this cell suspension were incubated with 5 µl annexin V–FITC and 10 µl of 50 µg/ml PI for 15 min at room temperature in the dark. Cells were analyzed by flow cytometry (Coulter EPICS XL-MCL; System II; Beckman Coulter, Krefeld, Germany) within 30 min.

Cell proliferation and viability assay

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

Coculture of multiple myeloma cells and bone marrow stromal cells

After 30 min adherence of 0.5×10^5/ml myeloma cells on a stromal cell layer, agents were added to the culture. After coincubation, myeloma and bone marrow stromal cells were detached by pipetting vigorously and by using a cell scraper. Myeloma cells were identified by FITC–labeled CD38 and/or CD138, and within the myeloma gate cell death was determined by evaluation of PI staining.

Statistics

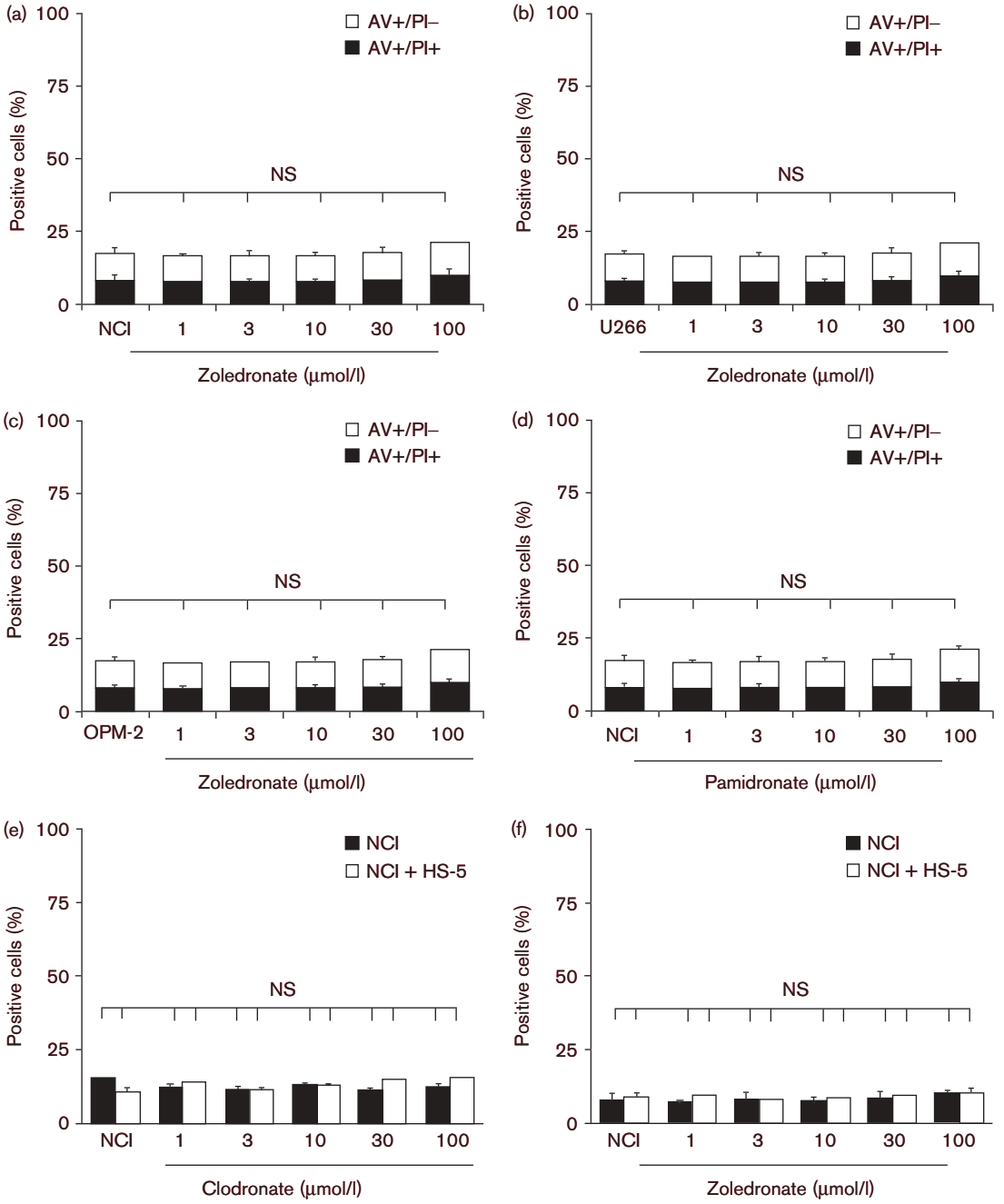
Mean values with standard deviations from representative experiments are shown in the figures. Kruskal–Wallis one-way analysis of variance on ranks was used to determine the statistical significance of treatment results. The pairwise multiple comparison procedure was performed according to the Student–Newman–Keuls method and the comparison versus control according to Dunn's method. Values of $P < 0.05$ were considered statistically significant.

Results

Antimyeloma activity of zoledronate

Contradictory data exist in the literature regarding the potency of zoledronate to induce apoptosis in multiple myeloma cells. This may be due to the selection of concentrations and incubation periods of the drug, the cell lines, and the methods to determine cell death and/or apoptosis. Therefore, it was deemed necessary to evaluate the antimyeloma activity of zoledronate in our experimental model. We chose annexin V/PI staining to determine cell death and early events of apoptosis, and used RPMI-8226, U266, OPM-2 and NCI-H929 human myeloma cell lines. We defined 100 µmol/l as the maximal concentration because peak serum levels of zoledronate are low and we assumed that millimolar concentrations are not achievable in humans, even in the bone marrow (see Discussion). Three multiple myeloma cells lines were incubated over 48 h with increasing concentrations of zoledronate from 1 to 100 µmol/l and cell death/apoptosis was determined by annexin V/PI staining. Figure 1 (a–c) shows that zoledronate is not able to substantially induce apoptosis in myeloma cells under these conditions. To exclude substance-specific resistance, we repeated the experiment with first-generation and second-generation bisphosphonates, clodronate and pamidronate using NCI-H929 cells, and similar results were obtained (Fig. 1d and e). We conclude that the class of bisphosphonates is without substantial antimyeloma effect in the chosen in-vitro model. Furthermore, reflecting the complex biology of multiple myeloma in the bone marrow microenvironment, we have repeated

Fig. 1



Zoledronate has no substantial proapoptotic antimyeloma effects at low concentrations up to 100 μmol/l. NCI-H929 (NCI), U266 and OPM-2 multiple myeloma cells were treated for 48 h with increasing concentrations of zoledronate, as indicated. Apoptosis was determined by flow cytometry after annexin V (AV)–fluorescein isothiocyanate and propidium iodide (PI) staining. Mean values and standard deviations are shown. *Indicates statistical significance ($P<0.05$). (a) Effect of zoledronate on NCI-H929. (b) Effect of zoledronate on U266. (c) Effect of zoledronate on OPM-2. (d) Effect of pamidronate on NCI-H929. (e) Effect of clodronate on NCI-H929 in the absence and presence of HS-5 bone marrow stromal cells. (f) Effect of zoledronate on NCI-H929 in the absence and presence of HS-5 cells. NS, not-significant.

the experiments in the presence of HS-5 bone marrow stromal cells. Again, no proapoptotic effects of clodronate and zoledronate were observed (Fig. 1e and f), even in coculture.

As mentioned above, bisphosphonates are inhibitors of the mevalonate pathway and inhibition of the mevalonate pathway by statins overcomes primary, adhesion-mediated drug resistance in multiple myeloma [13].

Although bisphosphonates did not induce apoptosis when given as a single agent, we tested whether 100 μmol/l zoledronate might overcome cell-adhesion-mediated drug resistance in an NCI-H929/HS-5 coculture model. Figure 2(a and b) shows that zoledronate does not reduce this primary type of drug resistance in the context of either melphalan or bortezomib treatment. Furthermore, this was not specific for zoledronate, as experiments using clodronate show induction of apoptosis by melphalan independent of prior clodronate exposure (Fig. 2c). Interestingly, there is some increase in apoptosis by addition of zoledronate to melphalan, which is of similar degree in monoculture and coculture, and which reaches statistical significance. This additional apoptosis, however, was not observed with clodronate or bortezomib.

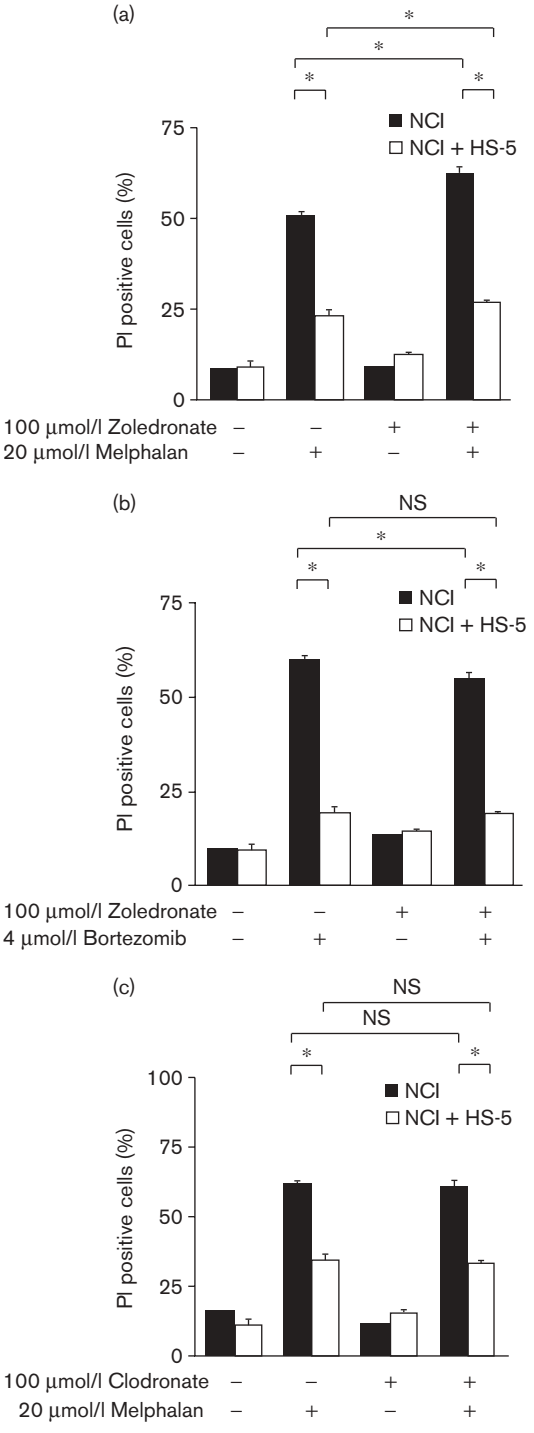
Antimyeloma activity of simvastatin

HMG-CoA reductase inhibitors like simvastatin have been shown to induce apoptosis and to inhibit proliferation of multiple myeloma cells. Furthermore, statins were found to overcome primary, cell-adhesion-mediated drug resistance. Figure 3(a) demonstrates that simvastatin significantly inhibits proliferation, even at very low concentrations of 1 μmol/l. As mentioned above, multiple myeloma is a disease of the bone marrow and the interaction of multiple myeloma cells with bone marrow stromal cells is crucial for the pathogenesis. Stromal cells support growth and survival of myeloma cells by secretion of cytokines like interleukin-6. Furthermore, direct cell–cell contact protects myeloma cells from drug-induced cell death. We have shown previously that compounds like dexamethasone, melphalan, treosulfan or doxorubicine induce significantly less apoptosis in the presence of direct cell–cell contact with stromal cells. This cell-adhesion-mediated drug resistance can also be demonstrated for the novel antimyeloma compound bortezomib (Fig. 3b). In contrast, simvastatin, as a single agent, induces apoptosis in monoculture of multiple myeloma cells and also overcomes primary, adhesion-mediated drug resistance. Therefore, we hypothesized that statins may induce apoptosis in the multiple myeloma/bone marrow stromal cell coculture that is not affected by the mechanisms of cell-adhesion-mediated drug resistance. Figure 3(c–e) demonstrates that in three different myeloma cell lines simvastatin potently induces apoptosis even in the presence of HS-5 stromal cells. To our surprise, we repeatedly observed a trend towards a higher cell death in coculture than in monoculture at intermediate concentrations in NCI-H929 cells (Fig. 3c). This effect, however, was less pronounced or absent in the other cell lines.

Antimyeloma activity of the combination of zoledronate and simvastatin

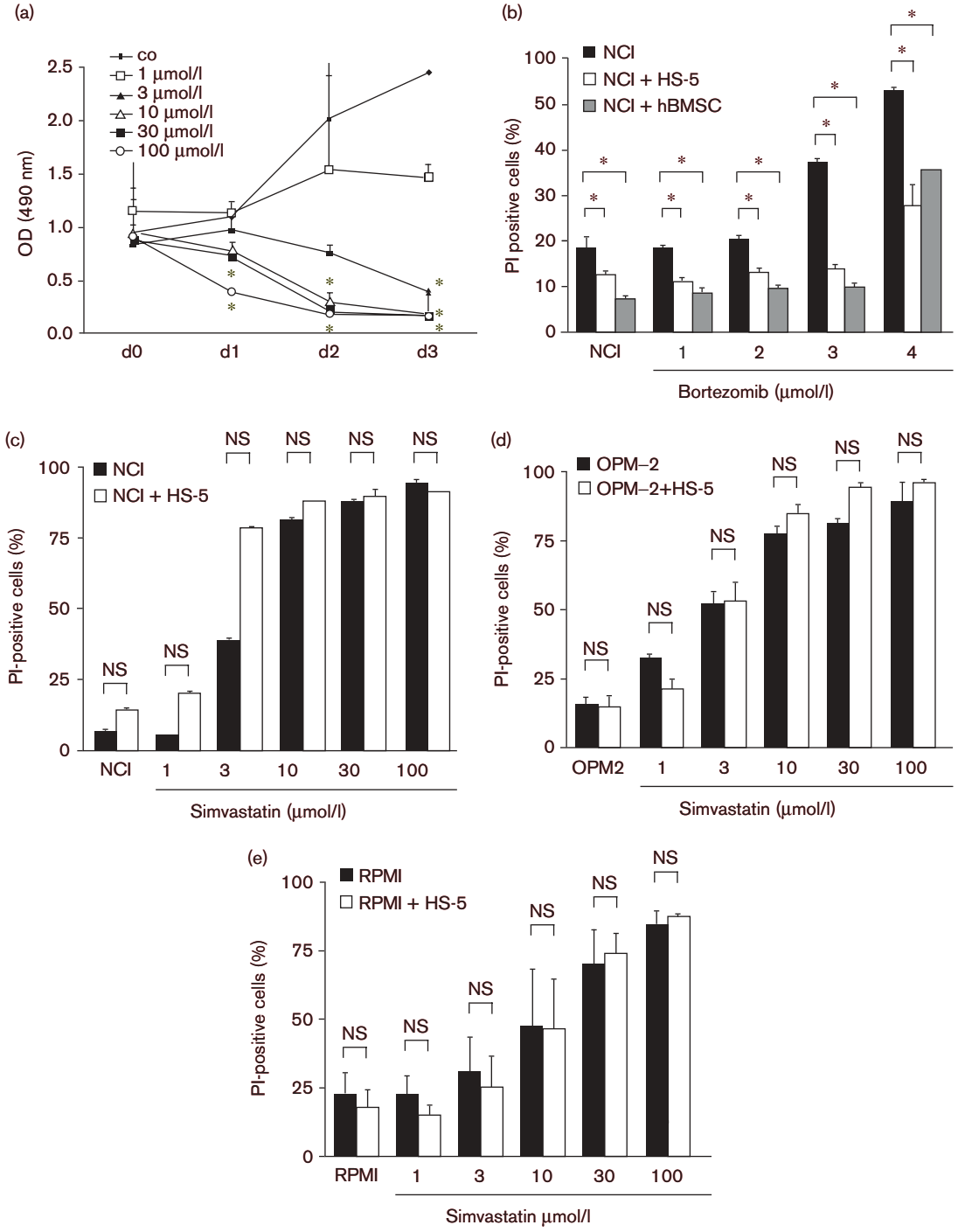
As we and others have shown, both statins and bisphosphonates have antimyeloma effects, but concentrations needed *in vitro* for biological effects do not seem to be

Fig. 2



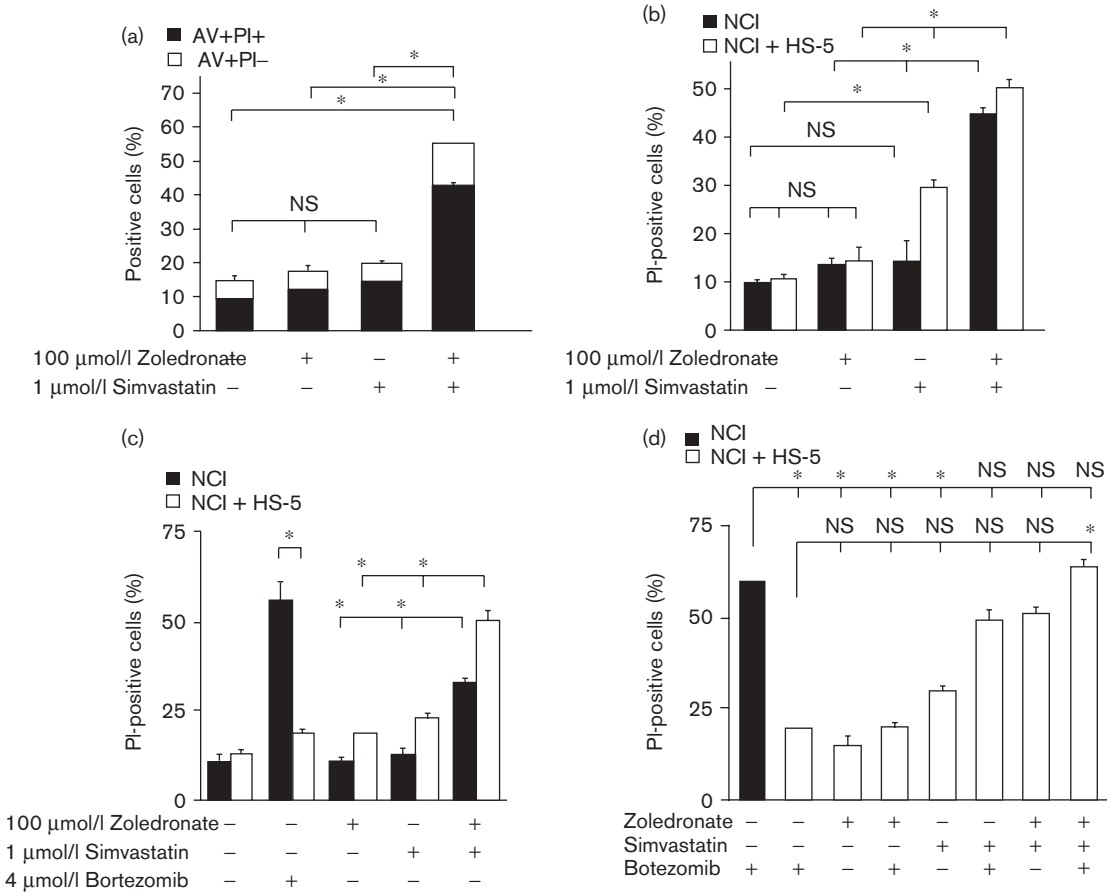
Zoledronate does not overcome cell-adhesion-mediated drug resistance at low concentrations up to 100 μmol/l. NCI-H929 myeloma cells were grown in the absence or presence of HS-5 bone marrow stromal cells for 48 h. Melphalan, bortezomib, zoledronate and clodronate were added as indicated. Myeloma cells were identified by CD38–fluorescein isothiocyanate staining and cell death was determined by propidium iodide (PI) uptake. Mean values and standard deviations are shown. *Indicates statistical significance ($P<0.05$). (a) Zoledronate does not overcome melphalan resistance. (b) Zoledronate does not overcome bortezomib resistance. (c) Clodronate does not overcome melphalan resistance. NS, not-significant.

Fig. 3



Simvastatin induces apoptosis without signs of adhesion-mediated drug resistance. NCI-H929 (NCI), RPMI-8226 (RPMI) and OPM2 multiple myeloma cells were incubated with increasing concentrations of simvastatin. (a) Cell viability was determined by water-soluble tetrazolium salt-1 assay at day 0 (d0), day 1 (d1), day 2 (d2) and day 3 (d3) of simvastatin incubation. Optical density (OD) was determined at a wavelength of 490 nm. Mean values and standard deviations are shown. *Indicates statistically significant results ($P<0.05$). (b) NCI-H929 (NCI) cells were grown in the presence or absence of bone marrow stromal cells, either HS-5 cell line or primary human bone marrow stromal cells (hBMSC) from patients. Cultures were treated for 48 h with bortezomib at the indicated concentrations. Myeloma cells were detected by CD38–fluorescein isothiocyanate staining and cell death was determined by propidium iodide (PI) uptake. (c) NCI-H929 (NCI), (d) OPM-2 and (e) RPMI-8226 (RPMI) cells were treated for 48 h with increasing concentrations of simvastatin in the presence or absence of HS-5 stromal cells, and PI uptake was measured. NS, not-significant.

Fig. 4



Zoledronate and simvastatin synergistically induce apoptosis and overcome cell-adhesion-mediated drug resistance. (a) NCI-H929 cells were treated for 48 h with zoledronate and simvastatin, as indicated. Apoptosis was determined by flow cytometry after annexin V (AV)–fluorescein isothiocyanate and propidium iodide (PI) staining. Mean values and standard deviations are shown. Representative data of four independent experiments with similar results are shown. (b) NCI-H929 cells were treated for 48 h with zoledronate and simvastatin as indicated, in the presence or absence of HS-5 stromal cells. Cell death was determined by flow cytometry after PI staining. (c) NCI-H929 cells were treated for 48 h with zoledronate, simvastatin and bortezomib as indicated, in the presence or absence of HS-5 stromal cells. (d) NCI-H929 cells were treated for 48 h with zoledronate, simvastatin and bortezomib as indicated, in the presence or absence of HS-5 stromal cells. Cell death was determined by flow cytometry after PI staining. *Indicates statistically significant results. NS, not-significant.

achievable in humans. As both drugs are inhibitors of the mevalonate pathway, we hypothesized synergistic biological effects by sequential blockage of this pathway. Bisphosphonates are an integral part of myeloma therapy and statins are widely used for the prevention of cardiovascular events. In light of these considerations, we tested combinations of the two drugs for possible synergistic effects using concentrations achievable in patients.

Figure 4(a) demonstrates that at concentrations of zoledronate and simvastatin that are non–toxic for multiple myeloma cells, the combination of both significantly induces apoptosis in more than 50% of the cells. Furthermore, even in the presence of HS-5 bone marrow stromal cells zoledronate and simvastatin synergistically induce apoptosis (Fig. 4b). Again, there is a trend towards a higher sensitivity of myeloma cells to simvastatin in the coculture, which is not statistically significant in comparison with monoculture, but which is statistically significant in comparison with the untreated coculture control. The reason for the increased dependency of myeloma cells on the mevalonate pathway in coculture is yet unknown and needs to be examined in further studies. To ensure that myeloma cells properly adhere to stromal cells for maximal protective effects, we repeated the experiments with a bortezomib control clearly showing that this model conveys strong cell-adhesion-mediated drug resistance in the context of other cytotoxic compounds (Fig. 4c). Finally, we raised the question whether sequential inhibition of the mevalonate pathway by statins and bisphosphonates would synergistically overcome cell-adhesion-mediated

drug resistance (Fig. 4d). Bortezomib potently induces apoptosis in NCI-H929 myeloma cells, but in the presence of HS-5 bone marrow stromal cells, cell death is almost completely abrogated. Whereas zoledronate alone is not capable of overcoming drug resistance, simvastatin significantly restores bortezomib-induced toxicity. It is noteworthy that a combination of simvastatin and zoledronate achieves similar toxicity, and, most important, the combination of all three drugs completely overcomes adhesion-induced bortezomib resistance. We therefore suggest the introduction of simvastatin in myeloma therapy, with a thoughtful scheduling of the drugs.

Discussion

The mevalonate pathway – more precisely the activation of the acetyl-CoA/HMG-CoA/mevalonate/geranylgeranyl-pyrophosphate cascade – seems to be of central importance for antiapoptosis and drug resistance in multiple myeloma [13]. Inhibition of this pathway by either statins or bisphosphonates has been suggested to induce antimyeloma effects. The biological mechanism of statin-induced cell death shows all the characteristics of apoptosis [5]. We have previously shown that geranylgeranylation of small G proteins is the downstream effect of statin-based inhibition of cell-adhesion-mediated drug resistance [13]. Similarly, inhibitors of the geranylgeranyl transferase, not of the farnesyl transferase, induce apoptosis in several multiple myeloma cell lines [9]. From our own experience and from the review of the literature, we concluded simvastatin to be the most, or at least one of the most potent statins with regard to antimyeloma activity. In this work, we show for the first time that statin-induced apoptosis in multiple myeloma is not hampered by protective effects of bone marrow stromal cells. As, so far, cell-adhesion-mediated drug resistance is seen for all drugs commonly used in the therapy of multiple myeloma, the combination of cytotoxic drugs with simvastatin may be an important step towards both the reduction of the tumor burden, which causes the clinical symptoms, and the targeting of the self-renewing myeloma (stem) cells, which cause relapse. Our data are in accordance with previous work, which demonstrated that neither the addition of cytokines nor adhesion to fibronectin protects myeloma cells from statin-induced apoptosis [14]. In addition, even secondary-resistant myeloma cells are shown to be highly sensitive to statins [11], further suggesting the use of statins in addition to classical cytotoxic drugs. The drug concentration commonly used for *in vitro* experiments (100 µmol/l range), however, may not be representative of therapy in humans, as serum levels of simvastatin are in the low micromolar range [15]. Subsequently, 1 µmol/l simvastatin was chosen as maximum concentration for our further experiments.

Pamidronate and zoledronate are approved for the therapy of multiple-myeloma-associated skeletal events. The present data suggest zoledronate to be more potent. Furthermore, dose escalation above the standard 4 mg zoledronate seems to be possible. Peak serum levels of zoledronate in humans range at about 1 µmol/l after standard infusion of 4 mg over 15 min; 8 µmol/l is achievable after infusion of 16 mg over 15 min [16]. Owing to the mode of action, the local concentration of bisphosphonates in the bone may, however, be much higher. Concentrations up to 0.1–1 mmol/l (alendronate) have been suggested in clear zones between bone and osteoclasts [17]. From routine microscopic studies, it is well known that myeloma cells are not strictly localized in these clear zones between osteoclasts and bone, but scattered throughout the whole marrow. We have therefore chosen a maximum concentration of 100 µmol/l zoledronate for our experiments, which is in between the measured serum levels and the calculated concentrations in the acidity resorption zones. At these concentrations, we were not able to detect substantial antimyeloma effects *in vitro*. At first glance, this seems to be in contrast to previously published data. For example, Ural *et al.* [18] have observed decreased metabolic activity (3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltertrazolium bromide assay) of ARH-77 and RPMI-8226 multiple myeloma cells after incubation with zoledronic acid. In contrast, Aparicio *et al.* [19] reported that ARH-77 cells are resistant to pamidronate-induced apoptosis. Zoledronate was shown to be more potent, but ARH-77 cells were still resistant. Tassone *et al.* [20] showed that zoledronate significantly reduces myeloma cell (XG-1, XG-1a, U266, IM-9) proliferation (45%) after long-term (6 days) incubation and Corso *et al.* [21] showed that 72 h of zoledronate substantially induces cell death (3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltertrazolium bromide assay) in RPMI-8226 cells with a calculated LD_{50} of 62 µmol/l. Annexin V analysis of primary myeloma cells from three patients revealed only 8% apoptotic cells after 4 days of 100 µmol/l zoledronate. Zoledronate (3 days of 100 µmol/l), however, induces morphological changes of RPMI-8226 cells like cell shrinkage, nuclear condensation and fragmentation [22]. Interestingly, U266 proved to be completely resistant to bisphosphonate-induced apoptosis, which is in accordance with our results. In addition, bisphosphonate-resistant subclones were generated by long-term incubation. No cross-resistance to conventional cytotoxic drugs and, more important, to HMG-CoA reductase inhibition (mevastatin) in terms of inhibition of proliferation and induction of apoptosis was observed. Another study showed antiproliferative effects in LP-1, JJN-3 and OPM-2 cells [23] when concentrations up to 500 µmol/l were used. In this study, only zoledronate and not pamidronate induced apoptosis in human myeloma cell lines. Furthermore, in OPM-2 and LP-1 cells 100 µmol/l zoledronate was without substantial effect, and only 500 µmol/l induced apoptosis. Compar-

ably high doses of pamidronate and incadronate decreased proliferation of U266, RPMI-8226 and KPMM2 cells [24]. In U266, no reduction of proliferation could be observed up to 100 µmol/l, but at 500 and 1000 µmol/l proliferation was significantly reduced. Similar results were obtained for pamidronate in RPMI-8226 and KPMM2 cells. Using annexin V/PI assays 500 µmol/l pamidronate induced only annexin V positivity at 48 and 72 h, but without PI positivity. Accordingly, pamidronate only induces a slight decrease of the cell number (JJN-3, HS-Sultan, U266-B1) at 100 µmol/l (72 h) and clodronate has no antimyeloma activity *in vitro* [25]. At 500 µmol/l pamidronate, the number of HS-Sultan cells was reduced to about 70% of that of control, whereas about 80% JJN-3 and U266-B1 cells remained viable. Although YM529 strongly inhibited proliferation and induced apoptosis in myeloma cell lines (U266, HS-Sultan, RPMI-8226), incadronate only slightly reduced proliferation, and apoptosis was induced only at 500 µmol/l [26]. In summary, there are many conflicting in-vitro data about the antimyeloma activity of bisphosphonates, and the corresponding in-vivo concentrations are unclear. In-vivo data show that ibandronate is not able to induce apoptosis in myeloma cells in a murine model [27]. On the other hand, it was shown that pamidronate induces a slight increase of apoptotic myeloma cells in the bone marrow *in vivo* after standard infusion of pamidronate [28]. Furthermore, pamidronate, and to a greater extent zoledronate, induce plasma cell apoptosis of primary myeloma cells *in vitro* (100 µmol/l 24 h). To find out whether clinically relevant antimyeloma effects can be achieved by bisphosphonates is the purpose of ongoing studies. Only the results of randomized phase III studies will answer this question [29].

Two classes of inhibitors of the mevalonate pathway, however, are clinically available and both show some antimyeloma effects *in vitro* and *in vivo*. In our experiments, the HMG-CoA reductase inhibitor simvastatin and the bisphosphonate zoledronate synergistically induce apoptosis in multiple myeloma cells. Importantly, this effect is not hampered by bone marrow stromal cells in terms of cell-adhesion-mediated drug resistance. The combination of both completely overcomes cell-adhesion-mediated drug resistance of bortezomib. In summary, we suggest that by sequential blockage of the pathway by using the combination of both compounds at relatively low concentrations the problem of (too) low serum and tissue concentrations of statins and bisphosphonates as single agents can be circumvented. Furthermore, induction of apoptosis independent of cell-adhesion-mediated drug resistance is only one part of the antimyeloma action, but overcoming resistance to classical antimyeloma drugs like bortezomib is possible as well. Therefore, we provide the rationale for clinical trials that test the combination of all three – conventional drugs, bisphosphonates and statins – in the therapy of multiple myeloma.

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