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ABD56 causes osteoclast apoptosis by inhibiting the NFκB and ERK pathways

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ABSTRACT

We have previously shown that the biphenylcarboxylic acid butanediol ester (ABD56) inhibits osteoclast formation and activity *in vitro* and *in vivo*. However, the mechanism of action of this compound is unknown. ABD56 inhibited osteoclast formation and caused osteoclast apoptosis, but had no effects on osteoblasts or macrophages. As the NFκB and MAPK pathways are essential for osteoclast formation and survival, we studied the effects of ABD56 on these pathways. ABD56 caused phosphorylation of p38, JNK and nuclear translocation of *c-jun* in osteoclasts. ABD56-induced apoptosis was prevented by the caspase inhibitor zVAD-fmk but was not prevented by the p38- or JNK-inhibitors. ABD56 completely abolished RANKL-induced IκB and ERK1/2 phosphorylation. Increasing the amount of RANKL partially rescued ABD56-induced apoptosis, indicating that the apoptosis is most probably due to the inhibition of survival signals such as ERK and NFκB, rather than activation of the p38 or Jnk MAPK pathways.

Bone is a dynamic tissue that is constantly renewed and adapted by bone remodelling. The two stages in this process, osteoclastic bone resorption which is followed by osteoblastic bone formation, are tightly regulated, and in health the balance between resorption and formation is equal, thereby maintaining bone density and bone strength. In pathological conditions such as osteoporosis, Paget's disease and inflammatory disease, the balance is shifted towards bone resorption, leading to bone loss, and an increased risk of fracture. The main therapeutic strategy used in these conditions, is the use of anti-resorptive drugs such as bisphosphonates [1,2].

Osteoclasts are derived from haematopoietic stem cells, and are closely related to the macrophage lineage [3]. The major signalling molecule directing osteoclast differentiation is the receptor activator of NFκB ligand (RANKL) [4]. This cytokine binds to RANK expressed on osteoclast precursors and induces activation of the NFκB and AP-1 signalling cascades. This pathway is essential, as mice lacking critical molecules in this pathway, such as RANKL, RANK, TRAF6, c-fos or NFκB subunits, develop osteopetrosis due the absence of osteoclasts [5–9].

We have previously reported that the butanediol ester of biphenylcarboxylic acid (ABD56) and related compounds inhibit osteoclastic bone resorption by inhibiting osteoclast formation and inducing osteoclast apoptosis [10]. However, the mechanism by which ABD56 induces osteoclast apoptosis is currently unknown. In this paper we studied the mechanism of action whereby ABD56 induces osteoclast apoptosis.

Methods

Osteoblast-bone marrow co-culture. Osteoclast formation and activity was studied using mouse osteoblast-bone marrow co-cultures as previously described [11]. Briefly, osteoblasts were isolated from the calvarial bones of 2-day-old mice by sequential collagenase digestion as described by van 't Hof et al. [12] and cultured in α-MEM supplemented with 10% FCS and penicillin/streptomycin cocktail at 37 °C in 5% CO_2. Bone marrow cells were flushed out of the long bones of 3–5 month old mice and erythrocytes were removed by Hypaque-Ficoll density gradient centrifugation. Osteoblasts and bone marrow cells were plated onto dentine slices in 96-well tissue culture plates at 8×10^3 cells/well and 2×10^5 cells/well respectively, in 150 μl of α-MEM supplemented with 10% FCS, penicillin/streptomycin and 10 nM 1,25-dihydroxy-vitamin D3. Test substances and IL-1α (10^4 U/ml) were added on day 7 and the cultures were terminated on day 10.

Mouse osteoclast generation. Bone marrow cells were isolated as described above, re-suspended in α-MEM supplemented with 10% FCS and penicillin/streptomycin, and cultured in one 10 cm petri dish per mouse for 3 days at 37 °C in the presence of 100 ng/ml M-CSF. Subsequently, the petri dish was washed with PBS, and the adherent cells harvested using trypsin digestion. The cells were resuspended in culture medium supplemented with 100 ng/ml RANKL and 25 ng/ml M-CSF, at a density of 4×10^4 cells/ml, and 125 μl per well of this suspension was seeded 96-well plates. The cells were cultured for 7 more days, with medium changes after 2 and 4 days.

Rabbit osteoclast generation. Rabbit bone marrow cells were flushed out of the long bones of newborn rabbits as previously described by David et al. [13] and plated onto 6-well tissue culture plates in 2 ml of α-MEM supplemented with 10 nM

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1,25-dihyroxyvitamin D3, 10% FCS and penicillin/streptomycin at a seeding density of 1×10^6 cells/well. Drugs were added on day 7 and the culture was terminated on day 9.

Tartrate-resistant acid phosphatase (TRAcP) staining. Osteoclasts were identified by staining for TRAcP essentially as described by van 't Hof et al. [14]. Briefly, at the end of the culture period dentine slices with adherent cells were fixed in 4% paraformaldehyde, washed with PBS, and incubated with naphthol-ASBI-phosphate, pararosanilin and tartrate in acetate buffer (30 mM) at 37 °C for 45 min. TRAcP positive cells with three or more nuclei were considered to be osteoclasts.

Quantification of resorption pits. After counting adherent osteoclasts, dentine slices were immersed in 20% sodium hypochlorite to remove the cell layer. Resorption pits were visualised by reflected light microscopy and a Diagnostic Instruments Insight camera coupled to an Image Analysis system. The area resorbed was quantified by Image Analysis using custom software developed using Aphelion ActiveX objects (ADCIS, France) [15].

Alamar Blue viability assay. Osteoblasts and M-CSF dependent bone marrow macrophages were isolated as described above and plated in 96-well plates at 10^4 cells per well in 100 μl α-MEM supplemented with 10% FCS and penicillin and streptomycin, and grown overnight. The next day drugs were added to the cultures, and culture was continued for another 48 h. At the end of the culture period cell survival was determined by adding 10 μl AlamarBlue reagent per well, incubating the cells for a further 3 h and measuring viability as fluorescence (ex. 530 nm, em. 590 nm) using a Biotek Synergy HT platereader.

Western blotting. Cells were washed in PBS and homogenized in lysis buffer containing PBS, 0.1% (w/v) sodium dodecyl sulphate (SDS), 0.5%(w/v) sodium deoxycholate and 2% (v/v) protease inhibitor cocktail (Sigma, Dorset, UK). Samples were spun at 12,000 rpm for 10 min at 4 °C, the supernatant was collected and protein concentration was determined using a Bio-Rad protein assay kit. Samples (20–50 μg lane) were run on a 10% acrylamide gel and blotted onto a nylon membrane. The membranes were washed in Tris buffered Saline (TBS) and incubated overnight at 4 °C with one of the following; actin (1:1000 dilution; Sigma, Poole, UK), inactive (p-30), cleaved (p-19) caspase-3, phospho-IκBα, total IκBα, phospho-IKK, total IKK, phospho-p42/44, total p42/44, phospho-P38, total P38, phospho-SAP/JNK, total JNK (Cell Signalling Technology, USA, 1:1000 dilution). Subsequently, the membranes were washed in Tris buffered Saline (TBS) and incubated with the appropriate secondary antibodies coupled to HRP, washed in TBS again, and bands visualised using chemiluminescence (Amersham, UK) on a Syngene GeneGnome imaging system.

Apoptosis assays. For morphological assessment of apoptosis, cultures were treated with ABD056 for 48 h, fixed in 4% formaldehyde, washed in PBS and stained with 1 μg/ml 4,6-diamidino-2-phenylindole (DAPI) for 3 min prior to analysis by fluorescence microscopy. Cells were scored as apoptotic on the basis of nuclear morphology. An average of 6 microscopic fields per treatment group was analysed at 200× magnification. For the assessment of caspase-3 activation, inactive (p-30) and cleaved (p-19) caspase-3 expression were assessed by Western blotting as described above.

Statistical analyses. Statistical analyses were performed using SPSS for Windows version 11. Significant differences between groups were determined by ANOVA. If more than two groups were compared, significant differences were determined using the Bonferroni correction. All data are presented as means ± SEM unless stated otherwise. Values of p less than 0.05 were considered significant.

Results

Osteoclast formation and bone resorption were inhibited by ABD56 in a dose dependent manner in both bone marrow-osteoblast co-cultures and in RANKL-stimulated bone marrow macrophage cultures, whereas the control compound ABD34, which lacks the alkyl side chain and terminal hydroxyl group essential for the activity of ABD56, had no inhibitory effect (Fig. 1A–C). In contrast, ABD56 had no effect on osteoblast (Fig. 1D) or M-CSF dependent macrophage viability (Fig. 1E).

Normally, osteoclasts display a flattened morphology when cultured on plastic tissue culture dishes. However, osteoclasts in cultures treated with ABD56 were shrunken and would detach from the surface, and exhibited apoptotic bodies. These observations

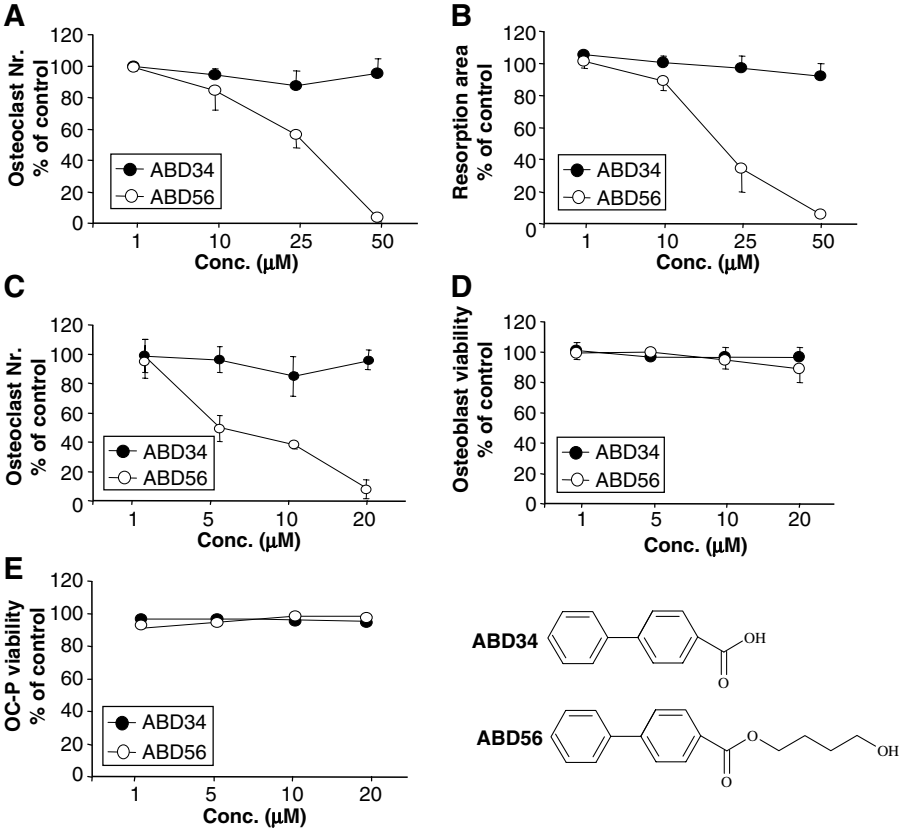

Fig. 1. ABD56 inhibits osteoclast formation and function but not osteoblast and macrophage viability. (A, B) Bone Marrow-osteoblast co-cultures were treated with vehicle (0.1% DMSO), ABD34 or ABD56 for 48 h. (C) RANK-L and M-CSF generated Osteoclast cultures were treated with ABD34 or ABD56 for 24 h. Multinucleated osteoclasts (A, C) were visualised by TRAcP staining and the resorbed area (B) was measured using reflective microscopy. (D, E) M-CSF dependent bone marrow macrophages (OC-P) and primary mouse osteoblasts were treated with vehicle (0.1% DMSO), ABD34 or ABD56 for 24 and 48 h, respectively. Macrophage and osteoblast viability was assessed by AlamarBlue assay. Data are means ± SEM of at least three independent experiments (n = 3–5), each performed in quintuplicate.

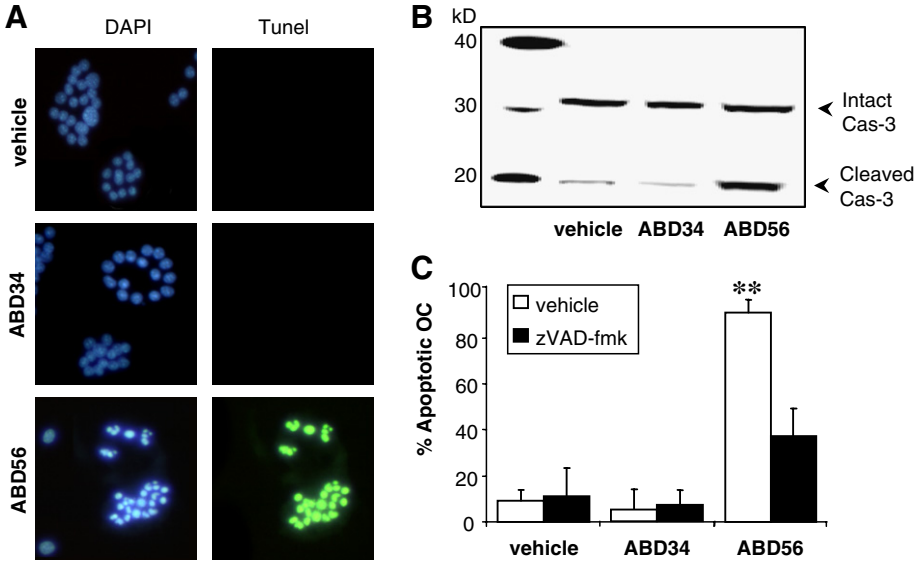

Fig. 2. ABD56 induces apoptosis in osteoclast cultures in vitro. Osteoclasts were treated with indicated agent (50 μM) for 48 h. (A) Microphotograph of osteoclasts stained with DAPI and DNA fragmentation analyzed by TUNNEL staining. Note the condensed nuclear morphology and intense TUNNEL stain in the ABD56 treated osteoclasts. (B) At the end of the treatment period, the cells were lysed and the presence of activated caspase-3 (cleaved Cas-3) analyzed by Western blotting. (C) OC were treated with the agents indicated in the presence or absence of the caspase inhibitor zVAD-fmk (20 μM) Apoptotic cells were visualised by DAPI staining and cells with clearly condensed nuclei were counted and expressed as a percentage of TRAcP positive MNCs ($^{**}p < 0.001$).

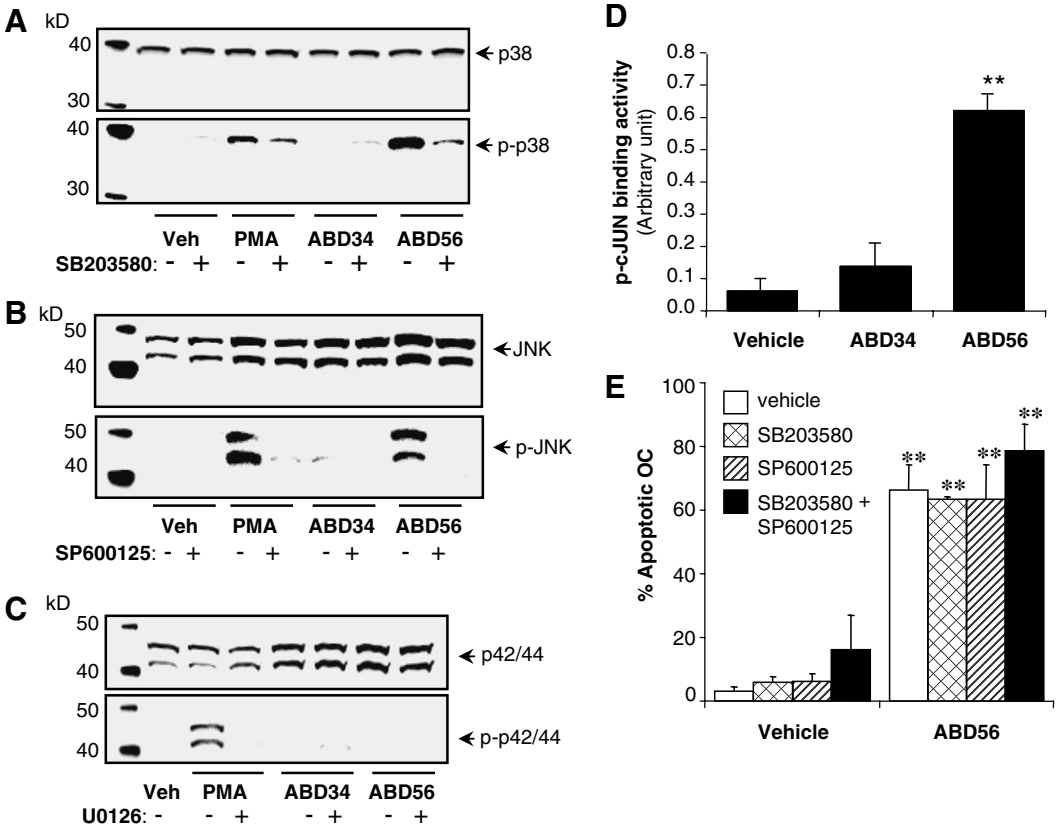

Fig. 3. ABD56 stimulates p38, JNK and c-JUN phosphorylation in osteoclasts, but ABD56-induced apoptosis is independent of p38 and JNK activation. (A–C) Osteoclast cultures were treated with vehicle (0.01% DMSO), SB203580 (20 μM), SP600125 (20 μM) or U0126 (20 μM) for 60 min and then stimulated with vehicle, PMA (20 μM), ABD34 (50 μM) or ABD56 (50 μM) for 30 min. Phosphorylation of p38 (A), JNK (B) and p42/44 (C) was analysed by Western blotting. Lane 1 on all blots is a molecular weight marker ladder. Identical experiments have been repeated three times. (D) Osteoclast precursors were treated with vehicle (0.01% DMSO), ABD34 (50 μM) or ABD56 (50 μM) for 120 min. Nuclear extracts were obtained and phospho-cJUN activity was measured using TRANS-AM AP-1 family Transcription factor assay kit ($n = 3$, $^{**}p < 0.001$). (E) Rabbit osteoclast were treated with vehicle (0.01% DMSO), SB203580 (10 μM), SP600125 (10 μM) or 5 μM of SB203580 and SP600125 for 60 min and then treated with vehicle or ABD56 (50 μM) for 48 h. Nuclei were stained using DAPI and apoptotic cells were identified by condensed nuclear morphology. Identical experiments have been repeated three times. ($n = 3$, $^{**}p < 0.001$ from vehicle control).

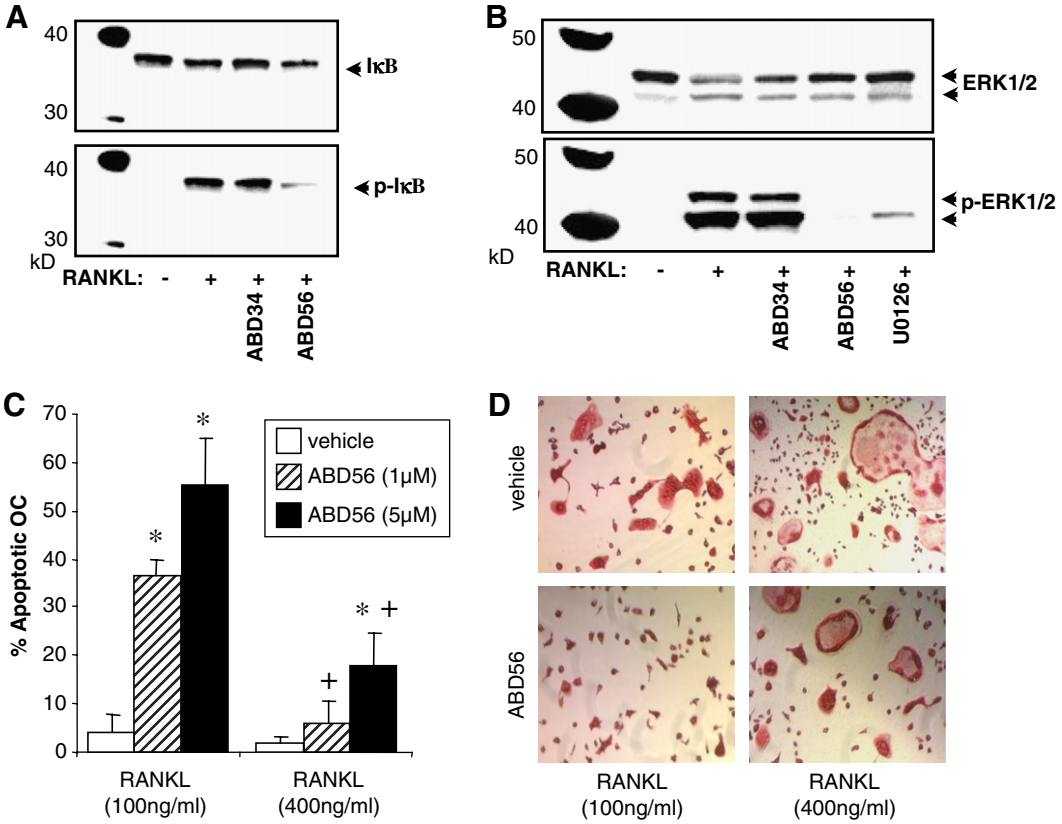

Fig. 4. ABD56 inhibits RANK-L-induced NFκB and ERK MAPK activation in osteoclast cultures. Osteoclast cultures were treated with vehicle (0.01% DMSO), ABD34 (50 μM), ABD56 (50 μM) or U0126 (20 μM) for 60 min and then stimulated with RANK-L (100 ng/ml) for 5 min. Phosphorylation of IkB or p42/44 ERK was assessed by Western blotting. Lane 1 on all blots contains a molecular weight marker ladder. Identical experiments have been repeated three times. (C) RANKL and M-CSF generated osteoclasts were treated with vehicle (0.01% DMSO) or ABD56 (1 and 5 μM) in the presence and absence of increasing concentrations of RANK-L (100 and 400 ng/ml). Multinucleated osteoclasts were visualised by TRAcP staining (as shown in d). Data are means ± SEM of at least three independent experiments ($n = 3$), each performed in quintuplicate. $^{*}p < 0.01$ from vehicle control, $^{+}p < 0.01$ from 100 ng ml RANKL.

suggested that ABD56 induces apoptosis in OC. To investigate this hypothesis, we performed assays for apoptosis in these cultures. As shown in Fig. 2A, ABD56-induced nuclear condensation and DNA fragmentation in osteoclasts. Furthermore, treatment with ABD56 led to activation of caspase-3 (Fig. 2B), a key step in the process of apoptosis. Indeed, treatment of the cultures with the caspase inhibitor zVAD-fmk strongly inhibited ABD56-induced osteoclast apoptosis (Fig. 2C), indicating the crucial role of caspase activation in this process.

The MAP kinase pathway has been shown to be involved in the regulation of apoptosis, and persistent p38 and JNK activation is known to stimulate apoptosis. Conversely activation of ERK is normally associated with cell proliferation and survival. We therefore studied the effects of ABD56 on these signalling molecules in osteoclasts. ABD56 did not cause ERK phosphorylation. However, both p38 and JNK phosphorylation were stimulated from 30 min after exposure to ABD56 (Fig. 3), and this persisted for at least 24 h (data not shown). The phosphorylation of p38 and JNK was inhibited by the p38 inhibitor SB203580 and the JNK inhibitor SP600125, respectively (Fig. 3). Activation of the JNK pathway was accompanied by an increase in DNA-binding of c-jun as shown in Fig. 3D. The inactive derivative ABD34 had no effect on MAP kinase or c-jun activation. The p38 inhibitor SB203580 and the JNK inhibitor SP600125 induced osteoclast apoptosis in mouse osteoclasts even in the absence of ABD56 (data not shown). However, as rabbit osteoclasts were resistant to the pro-apoptotic effects of SB203580 and SP600125, we used these cells to investigate whether p38 and JNK activation are necessary for ABD56-induced osteoclast apoptosis. As shown in Fig. 3E, ABD56 caused apoptosis in rabbit osteoclasts, and this was not prevented by incubation with SB203580, SP600125 or a combination of both.

As reported previously, ABD56 inhibits RANKL-induced NFκB activation. However, RANKL is also known to activate the ERK-MAP kinase pathway and subsequently c-fos. We therefore studied the effect of ABD56 on the ERK-MAP kinase pathway. As Fig. 4 shows, not only does ABD56 inhibit RANKL-stimulated NFκB activation, it also completely blocked ERK phosphorylation, and ABD56 was at least as potent as the specific ERK phosphorylation inhibitor U0126 (Fig. 4B). Increasing the amount of RANKL in osteoclast cultures partially prevented ABD56-induced osteoclast apoptosis (Fig. 4C and D), indicating that ABD56-induced apoptosis is most probably due to the inhibition of survival signals such as ERK- and NFκB, rather than activation of the p38 MAPK and Jnk pathways.

Discussion

The butanediol ester of biphenylcarboxylic acid ABD56 is a novel inhibitor of bone resorption, which acts by inducing osteoclast apoptosis. Non-specific effects are excluded by the fact that ABD56 did not affect related cells such as macrophages or osteoblasts. Here we have shown that ABD56-induced apoptosis is dependent on the activation of caspases as the caspase inhibitor zVAD-fmk strongly inhibited ABD56-induced apoptosis. Our experiments also showed that ABD56 inhibits RANKL-induced activation of the

ERK1/2 MAP kinase pathway as well as NFκB activation. NFκB and ERK are both cell survival signals and required for osteoclast formation and survival [5,6,16–18]. This suggests that the pro-apoptotic effects of ABD56 may be due at least in part to inhibition of NFκB and ERK signalling. In keeping with this hypothesis, ABD56-induced osteoclast apoptosis could be partially prevented by increasing the levels of RANKL.

ABD56-induced sustained phosphorylation of p38 and JNK, and sustained p38 and JNK activation can lead to apoptosis [19–23]. On the other hand, transient activation of the JNK and p38 pathways is crucial for osteoclast formation and survival [24–28]. Since p38 and JNK-inhibitors caused osteoclast apoptosis in our mouse osteoclast cultures (data not shown), we were unable to assess the importance of these pathways in ABD56-induced osteoclast apoptosis in these cells. However, in parallel we studied the effects of ABD56 on apoptosis in rabbit osteoclasts. We found that ABD56-induced apoptosis in rabbit osteoclasts but neither SB203580 nor SP600125 prevented this. These results indicate that activation of the p38 MAPK and JNK pathways are not essential for ABD56-induced apoptosis.

At present the exact molecular target for ABD56 is unknown. The fact that ABD56 inhibited both NFκB- and ERK 1/2-MAP kinase activation indicates that this compound acts in close proximity to RANK, probably at or before the point where the NFκB and ERK-pathways diverge. However, elucidating the exact molecular target will require extensive further experiments, which are beyond the scope of this paper.

In conclusion, ABD56 represents a novel class of bone resorption inhibitors that act by inducing osteoclast apoptosis through inhibition of RANKL-stimulated NFκB and ERK 1/2 activation. Its lack of effect on cells of the osteoblast lineage may make ABD56 of clinical value in the prevention and treatment of diseases characterized by increased osteoclastic bone resorption such as osteoporosis and Paget's disease of bone.

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