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Fas/CD95 is associated with glucocorticoid-induced osteocyte apoptosis

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Abstract

Prolonged use of glucocorticoids is associated with decreased bone formation, increased resorption and osteonecrosis, through direct and indirect effects on the activity and viability of bone effector cells, osteoblasts and osteoclasts, and osteocytes. This study has investigated molecular pathways implicated in Dexamethasone-induced apoptosis of osteocytes, using a cell line and primary chicken cells. MLO-Y4 osteocytes were pre-treated with several bisphosphonates representing a range of anti-resorptive activities and conformation/structure relationships, and were subsequently challenged with Dexamethasone. Apoptotic cells were detected at various times after treatment using morphological and biochemical criteria. Dex was shown to induce apoptosis associated with the Fas/CD95 death receptor and in a caspase 8 dependent manner. The apoptotic response was inhibited by all variants of the BP molecules, including those with reduced anti-resorptive activity, indicating that Dex-induced apoptosis is independent of anti-osteoclastic activity. Dex-induced apoptosis was associated with a transient increase in phosphorylated ERK 1/2 and was blocked by the ERK inhibitor UO126. In addition, both UO126 and BPs decreased localization of Fas to the cell membrane. ERK activation by PMA did not induce death or Fas upregulation, suggesting that Fas may be important for the induction of apoptosis and the existence of an additional factor activated by Dex which enables the cooperation between the Dex-activated

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ERK and Fas pathways, during apoptosis of osteocytes. Furthermore, upregulation of death and Fas was not accompanied by upregulation of FasL, pointing to the possible existence of FasL-independent Fas-associated death in these cells.

Keywords: Dexamethasone; Fas; ERK; Bisphosphonates; Osteocyte; Apoptosis

Introduction

Glucocorticoids (GCs) have been extensively used as anti-inflammatory agents due to their ability to modulate immune responses (Ashwell et al., 2000), commonly through activation of the Fas pathway, one of the best-characterised apoptotic pathways (Schmidt et al., 2001). Binding of FasL to FasR causes receptor oligomerisation and recruitment of an adapter protein, FADD, which interacts with caspase-8, initiating a caspase cascade leading to apoptosis. (Ashkenazi and Dixit, 1998).

As a side effect to their clinical applications, GCs are responsible for rapid and profound bone loss since they exert anti-mitotic effects on osteoblast precursor cells, induce apoptosis of mature osteoblasts and increase the resorptive activity of osteoclasts (Hamdy, 1997). Studies by Weinstein et al. identified the presence of a high proportion of apoptotic osteocytes in mice, compared to healthy controls, following chronic administration of prednisolone (Weinstein et al., 1998). It would be of benefit clinically to develop a concurrent prescription capable of reducing the unwanted side effects associated with GC-treatment.

The beneficial effects of Bisphosphonates (BPs) on bone have long been demonstrated against Paget's disease, post-menopausal osteoporosis and GC-induced osteoporosis, by decreasing the resorptive activity of osteoclasts (Rodan, 1998). BPs are classed as nitrogen-containing (such as PAM and ALN) and non N- BPs (such as CLO). In osteoclasts, N-BPs inhibit farnesyl diphosphate (FPP) synthase and prevent prenylation of small GTPases, such as Ras and Rho that are required for osteoclast polarization, resorption and cell survival, whereas non N-BPs are metabolized into cytotoxic analogues of ATP, that probably act as inhibitors of various ATP-dependent enzymes (Rodan, 1998; Rogers et al., 1999). Changes in structure and conformation have allowed the development of various N-BPs, which differ in their anti-resorptive activity since they differ in their ability to inhibit FPP synthase (Dunford et al., 2001).

In contrast to osteoclasts, the effect of BPs on osteocytes, which are considered the mechanosensors and transducers in bone, has not been well characterised. BPs have variously been shown to both decrease and increase ERK (Nishida et al., 2003; Plotkin et al., 1999). Studies by Plotkin et al have implicated the ERK1/2 pathway in the ability of BPs to prevent pro-apoptotic effects of Dex on MLO-Y4 osteocyte-like cells (Plotkin et al., 1999).

This study attempts to identify pro-apoptotic pathways employed by Dex as well as compounds that could potentially protect osteocytes from glucocorticoid-induced apoptosis. BPs and the MEK inhibitor UO126 were shown to protect against Dex-induced apoptosis, while upregulation of the Fas receptor appeared to be important in the induction of apoptosis.

Materials and methods

Cell culture

MLO-Y4 cell line

Unless otherwise stated, chemicals were purchased from Sigma, UK. MLO-Y4 murine osteocyte cell line was obtained from L. F. Bonewald (San Antonio, USA) and grown in collagen coated flasks in áMEM (Invitrogen, UK) supplemented with 5% fetal bovine serum, 5% newborn calf serum, 1% L-glutamine and 1% antibiotics, according to previously described methods (Kato et al., 1997). Cells were cultured until 90% confluence, before passage for experimental use.

Primary chicken osteocytes

Primary osteocytes were obtained and characterised as previously described (Nijweide and Mulder, 1986; Van der Plas and Nijweide, 1992; Aarden et al., 1996). Briefly, a mixture of osteoblasts and osteocytes was isolated by sequential collagenase-EDTA digestion from calvariae of 18-day-old chicken fetuses. Cell fractions were pooled and cultured for 1 day in MEM containing 2% chicken serum, 1.4 mM L-glutamine, 0.3 mM L-ascorbic acid (Merck, UK), 5.6 mM glucose (Invitrogen, UK), and 0.5 μg/ml gentamycin (Invitrogen, UK). Cell fractions were harvested by trypsin-EDTA, subjected to immunomagnetic isolation of osteocytes (OCY) by use of the chicken OCY-specific monoclonal antibody (MAb) OB 7.3, bound to magnetic beads (DYNAL, Oslo, Norway), which reacts specifically with OCY. A magnetic field was used to separate cells bound to beads, (1–8 beads/cell), used as isolated OCY. More than 95% of the cell population were OCY, as shown by staining with MAb OB 7.3. Then 2.5×10^4 OCY with beads were seeded onto glass slides and used for experiments the next day.

Cell treatment

For experimental manipulations, cells were plated in growth medium at a density of 1×10^4, in 24 multiwell plates for 24 hours, prior to experimentation. Experiments were carried out a minimum of 3 times, and each treatment group was represented by 3 wells in each independent experiment. Cells were observed in 3 fields per well (×20 magnification lens, approximately 40–100 cells per field) resulting in 9 fields per treatment group. Identical magnifications were used for all apoptosis estimates allowing similar numbers of cells to be counted per field in all experiments. For western blot analysis, cells were plated at a density of 1×10^5 in 60 mm petri dishes.

Induction of cell death

Cells were incubated in growth medium supplemented with 10^{-8} to 10^{-6} M Dex (Calbiochem, UK) and 0.4 μM to 0.4 mM H_2O_2 for 1–24 hours.

Prevention of cell death using BPs and inhibitors of intracellular signaling proteins

Osteocytes were pre-incubated with caspase 8 substrate II inhibitor Z-IETD-FMK (Calbiochem, UK) and caspase 3/7-selective inhibitors (GlaxoSmithKline, USA) at 10^{-6} M for 1 and 24 hours, to evaluate the

role of the Fas pathway in Dex-induced apoptosis. Cells were also pre-treated for 1 hour with PAM, ALN, CLO (kind gift form Prof. Mike Rogers, University of Aberdeen, UK) and the heterocyclic-containing N-BPs, NEII808 and NEII809 (Procter and Gamble, USA), at concentrations of 10^{-8} to 10^{-6} M, followed by Dex treatment. In addition, cells were pre-incubated for 30 minutes with UO126, a MEK 1/2 inhibitor (Promega, UK) at concentrations of 10 to 30 μM (Favata et al., 1998), SB203580 (Calbiochem, UK), a p38 inhibitor at concentrations of 5 to 15 μM (Cuenda et al., 1995) and with PMA at 2 ng/ml-200 μg/ml for 1 to 5 hours. All pre-treatment agents were maintained in cultures in the presence of Dex or H_2O_2.

Determination of apoptotic state

A range of techniques were used to determine the presence of apoptotic cells, for all the independent experimental culture setups, but only representative examples of each technique are shown throughout the manuscript:

1) DAPI staining for healthy and apoptotic cell morphology

Following treatment, cells were fixed in 4% paraformaldehyde, washed in PBS and air dried. Cells were then incubated with DAPI at 2.5 ng/ml for 10 minutes, washed in PBS and examined by fluorescence microscopy and digital image capture.

2) Acridine orange (AO) staining for healthy and apoptotic cell morphology.

Following treatment, cells were immediately incubated in Walpole's acetate buffer pH 4.2 (10:7, 1 M NaAc: 1 M HCl) for 5 minutes, followed by AO staining for 25 minutes. Cells scored as apoptotic were characterised by nuclear or cytoplasmic condensation, two or more nuclear fragments, single crescent-shaped nucleus or blebbing.

3) DNA fragmentation using in situ Nick Translation (NT)

Cells demonstrating DNA breaks were investigated in samples fixed in 4% paraformaldehyde, using a previously described DNA nick translation technique (Noble et al., 1995, 1997). Positive controls were established through pre-treatment with DNaseI at 0.2 mg/ml for 30 minutes. Cells were exposed to NT mixture which consisted of 3 μM Digoxigenin (DIG) labelled dUTP (DIG-11-dUTP), 3 μM each of dATP, dGTP, dCTP, 50 mM Tris HCl, pH 7.5, 5 mM $MgCl_2$ and 0.1 mM dithiotreitol and 0.5 μl/100 μl DNA polymerase for 1 hour at 37 °C, in a humidified chamber (Roche, UK). Wells were incubated for 1 hour at RT with fluorescein isothiocyanate (FITC)-labelled anti-DIG antibody and 5% normal sheep serum. Wells were then washed in PBS and counter stained with propidium iodide (PI). Cells containing fragmented DNA stained positive for FITC label and PI, as determined using fluorescence microscopy and digital image capture, based on 3 fields from a total of 3 wells per treatment.

4) Annexin-V-FITC Assay

During apoptosis, PS exposure on the outer leaflet of the membrane bilayer can be detected with fluorescently labelled Annexin V. Cells were incubated with Annexin-V-FITC at 1 μg/ml for 15 minutes at RT, followed by PI to identify necrotic cells in the culture. Fluorescence microscopy, allowed discrimination between viable (FITC negative, PI negative), apoptotic (FITC positive) or necrotic cells (FITC negative, PI positive).

Determination of Fas expression by immunocytochemical staining

Following incubation with various agents, cells were fixed in 4% paraformaldehyde and subsequently washed in PBS. Cells were incubated for 5 minutes with 0.1% SDS, and were washed

in PBS thoroughly. Cells were then blocked for 20 minutes in goat serum followed by 1 hour incubation with anti-Fas mAb at RT. After washing in PBS, cells were incubated with secondary anti-mouse FITC antibody for 1 hour and were counterstained with PI. Cells staining positive for FITC label and PI, were considered as cells expressing Fas on the plasma membrane, as determined using fluorescence microscopy and digital image capture, based on 3 fields from a total of 3 wells per treatment.

Reverse transcription-polymerase chain reaction

Total RNA was isolated from cultures of MLO-Y4 cells using RNA-B™ (Biogenesis) according to the manufacturers instruction. cDNA was synthesized from 3 μg of total RNA using oligo dT primers, and RNA was converted into cDNA by SuperScript II RNaseH$^-$ reverse transcriptase first strand synthesis system for RT-PCR (Invitrogen). The PCR reaction was performed using Qiagen Taq PCR core kit (10X reaction buffer, Taq 5 u/μl, Q buffer and dNTP 10 mM each) in a total of 25 μl reaction containing 5 μM each forward and reverse primers. Mouse Fas specific primers were designed against sequence accession number M83649 and mouse β-Actin specific primers against sequence accession number X03765 from HGMP database as shown below. The resulting PCR products for Fas and Actin were 220 bp and 290 bp respectively. The PCR reaction was carried out for 33 cycles with PTC-200 Peltier thermal cycler (MJ Research). PCR conditions were denaturation at 94 °C for 50 seconds, annealing at 50.5 °C for 1 minute and extension at 72° for 1 minute 30 seconds. The PCR products were analysed in 1.5% agarose gel containing ethidium bromide.

Primer Sequences:

Fas forward 5′ -CATGCTGTGGATCTGGGCTGT-3′
Fas reverse 5′ -GTGTACCCCCATTCATTTTGC-3′
Actin forward 5′ -CAAGGTGTGATGGTGGGAATG-3′
Actin reverse 5′ -GCTACGTACATGGCTGGGGTG-3′

The Accession number for the primers used for FAS ligand PCR is U10984. The identity of all PCR products was confirmed on DNA sequencing.

Western blot analysis of intracellular signalling proteins

Cells were maintained in identical culture conditions (presence of serum) to those used to study effects on apoptosis. Lysates were prepared (lysis buffer: 20 mM Tris-HCl, pH 7.5, 0.1% (v/v) Igepal, 6 mM sodium deoxycholate, 150 mM NaCl, 2 mM EGTA, 2 mM EDTA, 0.1 mM Na_3VO_4 and 20 mM NaF and a protease inhibitor cocktail tablet (Roche, UK)), and protein concentrations were estimated using a commercially available kit (Bio-Rad, UK). Lysates (30 μg/lane) were resolved on SDS-PAGE gels, transferred onto PVDF membranes and blocked with TBST solution (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20) supplemented with 3% BSA, and then hybridized with rabbit polyclonal antibodies against phospho-p44/42MAPK, phospho-MEK 1/2, phospho-cRaf, phospho-p90rsk and total MAPK p44/42 (New England Biolabs, UK). Proteins were detected using ECL reagents according to the manufacturers instructions. Blots were stripped (100 mM β-mercaptoethanol, 69 mM SDS and 62.5 mM

Tris-HCl, pH 6.7) and rehybridised with an antibody that recognises total p44/42 MAPK, to verify equal loading of samples.

Immunoprecipitation and western blot analysis of Fas

Protein extracts (1 mg) from cell lysates were diluted to 1 mg/ml and incubated with 15 μl of protein A agarose conjugate bead slurry (50% conjugate/ 50% PBS) and 4 μl of Jo2 hamster anti-Fas monoclonal antibody (BD Transduction Laboratories, USA) overnight at 4 °C. The agarose beads were collected by centrifugation at 4 °C, washed four times in lysis buffer and were then subjected to electrophoresis as described above. Proteins on PVDF membranes were hybridized with Jo2 antibody followed by secondary anti-hamster antibody (Abcam, USA) and were detected using ECL reagents.

Statistical analysis

All statistical analyses were performed using quantitative data analysis with SPSS release 11.5 for Windows. Analysis of Variance (ANOVA), Tukey test and Dunnett test were performed for comparison between the treatment groups. The Tukey test allows comparison of more than two means at once since this reduces the error associated with multiple t-tests (Zar, 1984). The Dunnett test allows comparison between the control mean and every other mean in the group (Zar, 1984). Results are expressed as means ± S.D. $p<0.05$ was considered to be statistically significant.

Results

Dexamethasone induces MLO-Y4 cell apoptosis in a time- and dose-dependent manner

MLO-Y4 osteocytes were cultured with Dex at $10^{-7}-10^{-5}$ M for various times between 1 and 24 hours (Fig. 1A and B). Apoptotic osteocytes appeared irregularly shaped and smaller in size, as shown by AO staining, while DAPI staining revealed chromatin condensation, shrinkage of nuclei and the fragmentation of the nuclear material into smaller blebs (Fig. 1C). Maximal levels of death accompanied by cell loss were reached at 5 hours of incubation with concentrations of 10^{-5} M and 10^{-6} M, whereas, at 24 hours, numbers of osteocytes in culture recovered somewhat, while apoptotic levels decreased, suggesting that Dex-induced death of osteocytes is transient (Fig. 1A and B). At concentrations of 10^{-7} M, Dex did not induce apoptosis in these cells. At 10^{-5} M a small proportion of cells were noted to have expanded and burst characteristics of necrosis. Based on the apoptotic criteria in response to the different concentrations and time points investigated, the dose of 10^{-6} M, which did not induce necrosis, was selected for future experiments, reaching a peak at 5 hours incubation.

Assessment of Dex-induced apoptosis by Annexin-V-FITC and nick translation assays

Early apoptotic features induced by Dex at 5 hours, were detected by both Annexin-V-FITC, which resulted in 12.35% ±1.14 osteocytes expressing PS (<1% necrotic cells identified by PI counter-

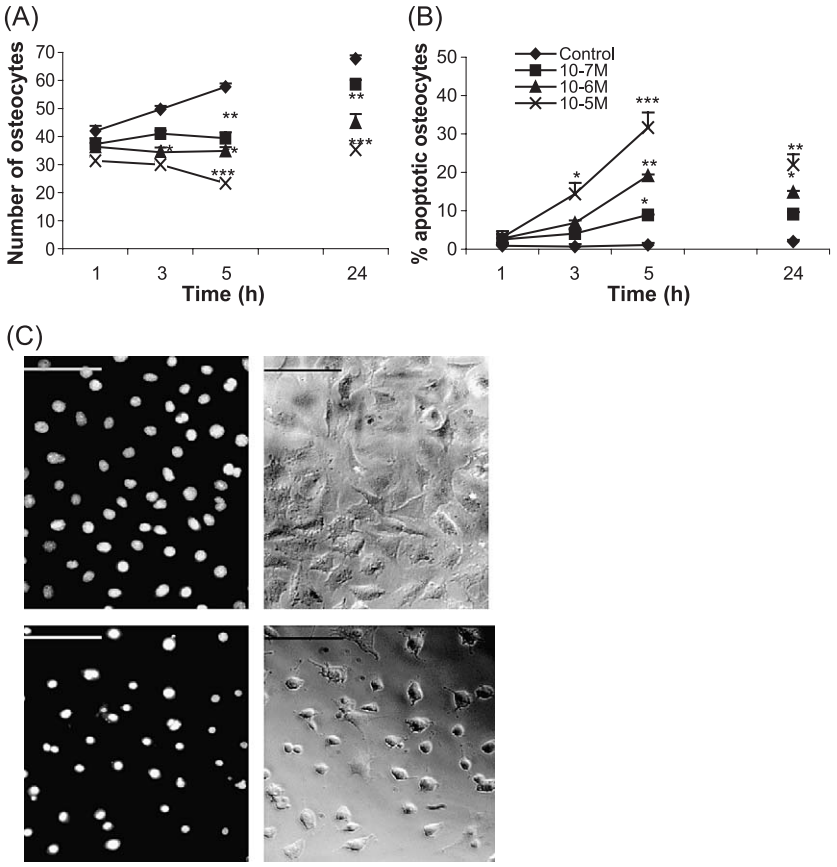

Fig. 1. Dexamethasone induces apoptosis in MLO-Y4 osteocytes in a time- and dose-dependent manner. MLO-Y4 cells were then stained with A.O. and examined by fluorescence microscopy to determine (A) Number of cellsand (B) Percentage of apoptotic osteocytes per number of cells ± S.D., in response to different doses of Dex and time. (C) Representative images of untreated (top panel) and Dex-treated MLO-Y4 osteocytes (bottom panel) at 10^{-6} M for 5 hours stained with DAPI. Bar = 5 μm. (*** = $p < 0.0001$, ** = $p < 0.001$, * = $p < 0.05$).

staining), and by Nick Translation assay, which resulted in 12.83% ±1.00 osteocytes positive for DNA breaks, compared to 1.33 ± 0.67 and 1.85 ± 1.38 in control cultures, respectively (data not shown).

Dexamethasone upregulates the Fas pathway

RT-PCR studies revealed that MLO-Y4 osteocytes express Fas receptor either in basal state or following treatment with Dex (Fig. 2A). However, FasL expression was not detected in both treated or untreated samples (data not shown). Immunocytochemistry showed that Dex increased the percentage of cells staining positive for Fas as early as 3 hours by 7-fold and at 5 hours by 22-fold compared to control (Fig. 2B and D). In addition, western blot analysis demonstrated time-dependent increase in Fas protein upon treatment with Dex (Fig. 2D).

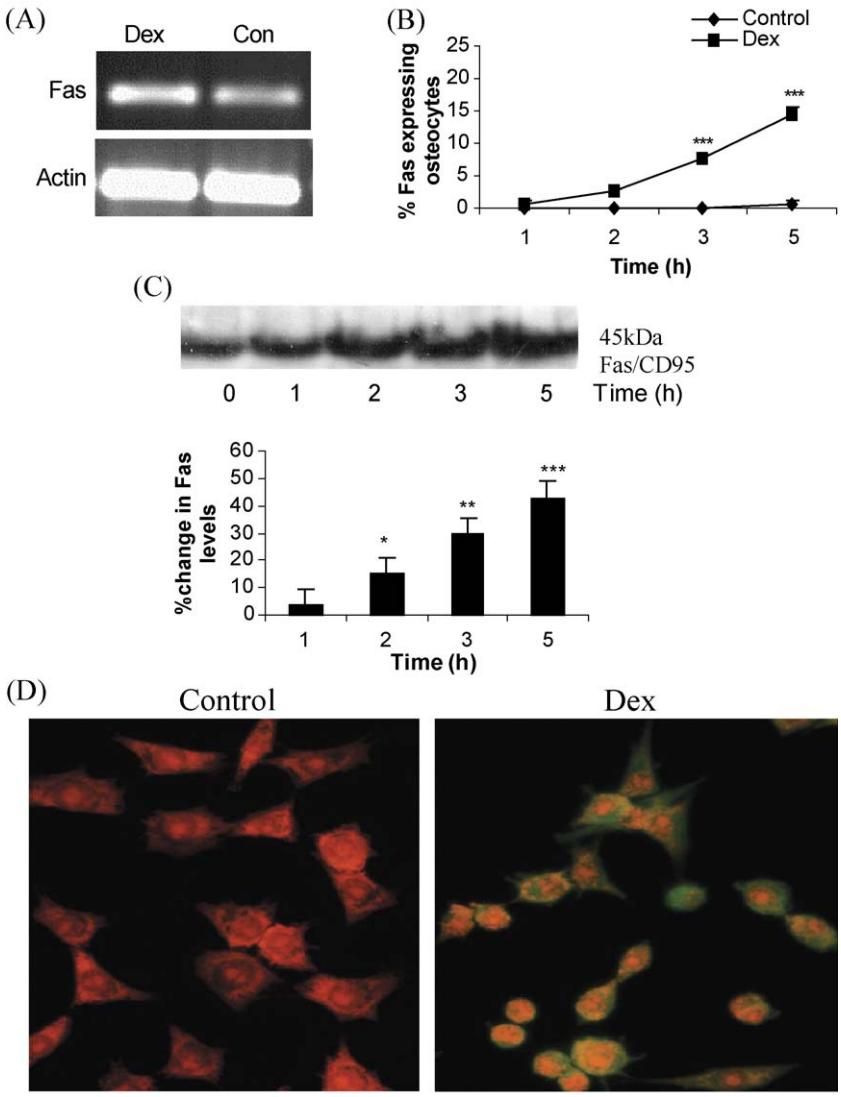

Fig. 2. Dex upregulates Fas expression in MLO-Y4 osteocytes. (A) Fas expression was confirmed using RT-PCR with RNA extracted from MLO-Y4 cultures treated with Dex. (B) Fas protein was detected following Dex treatment for 1 to 5 hours using a Fas mAb and fluorescence microscopy. Graph shows percentage of cells expressing Fas ± S.D. (C) Detection of Fas protein levels following Dex treatment for 1 to 5 hours using western blot analysis. Changes in band densitometry were quantified using NHI image analysis system and expressed as percentage change relative to zero time control ± S.D. (D) Representative images of fluorescence microscopy (20x magn.). Untreated osteocytes (left panel) and Dex-treated osteocytes (right panel). (*** = $p < 0.0001$, ** = $p < 0.001$, * = $p < 0.05$ compared to control).

The involvement of Caspase 8/FLICE, which lies downstream of Fas, was then investigated, in Dex-induced apoptosis. Pre-treatment with the caspase 8 inhibitor Z-IETD-FMK reduced the induction of death at 5 hours, up to 5-fold, compared to cultures treated with Dex, as shown by Annexin-V-FITC assay, suggesting that caspase 8 is involved in the apoptotic machinery

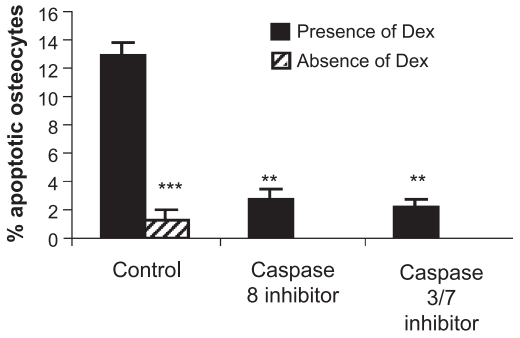

Fig. 3. Inhibitors of caspases 8 and 3,7 reduce pro-apoptotic stimuli induced by Dex. MLO-Y4 osteocytes were incubated with inhibitors of caspase-8 (Z-IETD-FMK) and a caspase 3/7 selective inhibitor for 1 hour, followed by Dex treatment for 5 hours. Cells were incubated with Annexin-V FITC and examined by fluorescence microscopy for PS detection. Graphs represent percentage of apoptotic osteocytes per number of cells ± S.D. (*** = $p < 0.0001$, ** = $p < 0.001$, * = $p < 0.05$).

activated by Dex in MLO-Y4 osteocytes (Fig. 3). In addition, pre-treatment with caspase 3/7 - selective inhibitors for identical times reduced the percentage of apoptotic osteocytes up to 6-fold (Fig. 3).

Bisphosphonates prevent Dex-induced apoptosis in MLO-Y4 osteocytes

BPs were used at concentrations that did not increase apoptosis above control levels (data not shown), to treat osteocytes prior to addition of Dex in cultures. Estimation of apoptosis showed that N-BPs (PAM, ALN and the heterocyclic-containing NEII808 and NEII809) and non N- BPs (CLO) at 10^{-8} M significantly decreased the pro-apoptotic effect of the corticosteroid at 5 hours incubation on osteocytes (Fig. 4).

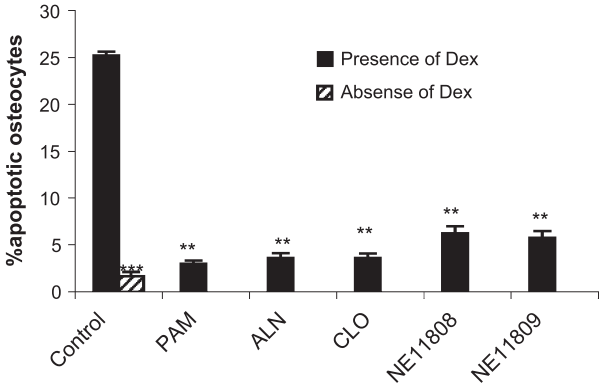

Fig. 4. BPs prevent MLO-Y4 osteocyte apoptosis induced by Dex. MLO-Y4 osteocytes were incubated with BPs at 10^{-8}M prior to Dex treatment, for 5 hours. Cells were stained with AO and examined by fluorescence microscopy. Graphs represent percentage of apoptotic osteocytes, per number of cells ± S.D. (*** = $p < 0.0001$, ** = $p < 0.001$, * = $p < 0.05$).

Inhibitors of MAP kinase signalling molecules prevent Dex-induced apoptosis in MLO-Y4 osteocytes

The effect of Dex on MLO-Y4 osteocytes was further characterised in the presence of protein inhibitors such as the MEK1/2 inhibitor UO126 and the p38 inhibitor SB203580. Dose response studies identified optimal concentrations of UO126, which did not significantly increase apoptosis above control levels either for the compound alone, or the vehicle in which it was delivered, within the range of concentrations known to inhibit ERK 1/2 (Favata et al., 1998) (Fig. 5). Quantification of apoptosis showed that concentrations of UO126 at 10 and 20 μM exerted protective effects on osteocytes following 5-hour incubation period with Dex (Fig. 5). In contrast, SB203580 at concentrations of 5 to 15 μM known to inhibit p38 (Cuenda et al., 1995), did not prevent GC-induced apoptosis, while doses above 10 μM induced significant apoptosis when added alone to osteocyte cultures (data not shown).

Primary cultures of chicken osteocytes were also used to observe the Dex induced death response in primary cells. Evaluation of apoptosis after a 5 hour-culture period, revealed that in a similar way to that seen in the MLO-Y4 cell line, both BPs and the MEK inhibitor UO126 were capable of blocking Dex-induced death in these primary cells (data not shown).

BPs and protein kinase inhibitors do not protect osteocytes from oxidant-induced death

To evaluate the role of the BP and ERK pathways in the induction of osteocyte apoptosis by other agents, MLO-Y4 cells were pre-treated with PAM and UO126 prior to their exposure to H_2O_2, at concentrations shown to induce apoptosis (0.08 mM to 0.4 mM). Examination of apoptotic morphology by AO staining revealed that PAM and UO126 did not protect MLO-Y4 osteocytes against H_2O_2 induced death stimuli (data not shown).

Dexamethasone activates the MEK/ERK protein signaling pathway

Incubation with Dex, increased the amount of activated ERK 1/2 protein in osteocytes, compared to vehicle, in a time dependent manner, as evidenced by western blot analysis, using an anti-

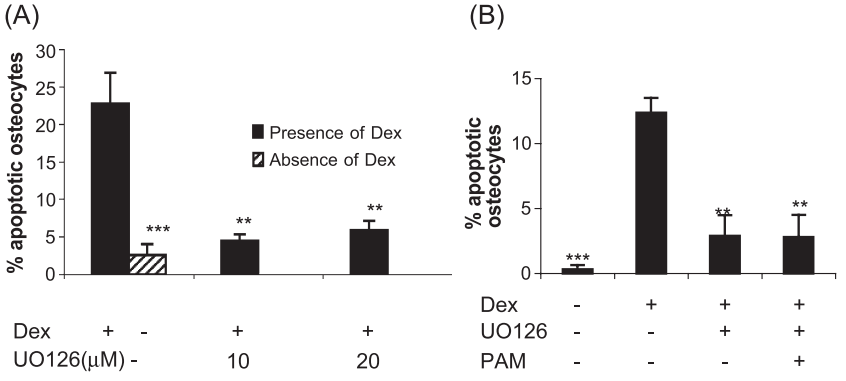

Fig. 5. The MEK 1/2 inhibitor UO126 prevents Dex-induced apoptosis. (A) UO126 prevents MLO-Y4 osteocyte apoptosis induced by Dex, in a dose dependent manner, and (B) in the presence of PAM. Cells were stained with AO and examined by fluorescence microscopy. Graphs represent the percentage of apoptotic osteocytes per number of cells ± S.D. (*** = $p < 0.0001$, ** = $p < 0.001$, * = $p < 0.05$).

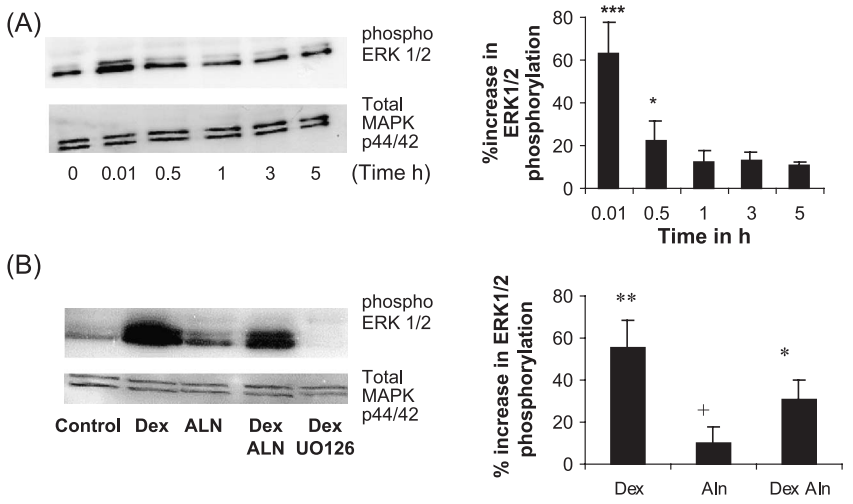

Fig. 6. (A) Dex activates the ERK 1/2 protein kinase, in a time dependent manner. ERK phosphorylation induced by Dex was statistically different from control during all time points investigated. (B) ALN reduces the Dex-induced ERK1/2 activation, at 5 minutes, whereas UO126 blocks Dex-induced ERK 1/2 activation. MLO-Y4 cell lysates were subjected to Western Blot analysis, using an anti-phospho MAPK p44/p42 antibody. The blots were stripped and reprobed with a total anti-MAPK p44/p42 antibody, to verify equal loading of samples. Changes in band densitometry were quantified using NHI image analysis system and expressed as percentage change relative to control samples, representing either vehicle control or zero time control ± S.D., from 3 independent blots. ** = $p < 0.001$, * = $p < 0.05$ compared to control, + = $p < 0.05$ compared to Dex treatment.

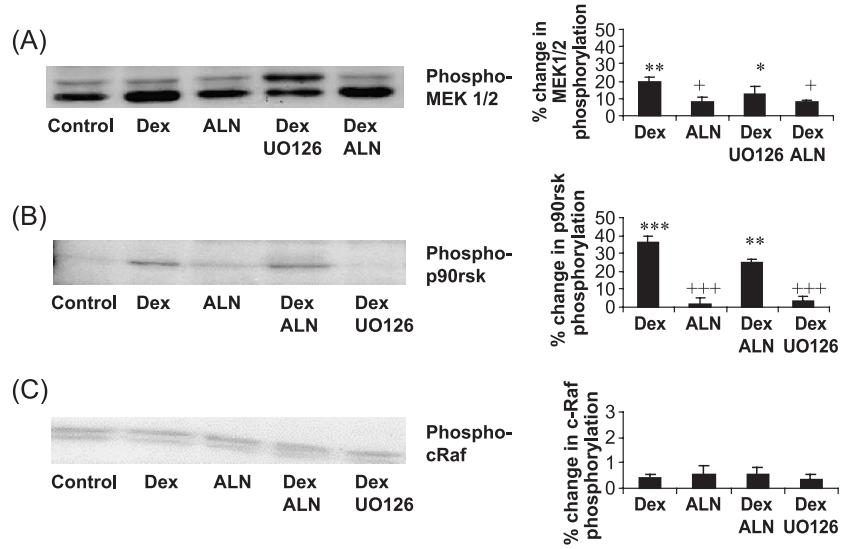

Fig. 7. Dexamethasone activates the ERK 1/2 pathway. Lysates prepared from MLO-Y4 cells treated with Dex, ALN and UO126, were subjected to Western Blot analysis, using (A) anti-phospho MEK 1/2 antibody, (B) anti-phospho p90rsk and (C) anti-phospho c-Raf antibody. Changes in band densitometry were quantified using NHI image analysis system and expressed as percentage change relative to vehicle sample ± S.D., from 3 independent blots. ***=$p < 0.0001$, **=$p < 0.001$, * = $p < 0.05$, compared to control +++ = $p < 0.0001$, + = $p < 0.05$ compared to Dex treatment.

phospho ERK 1/2 antibody (Fig. 6A). Activation of ERK 1/2 protein by Dex was acute, since it was detected as soon as 1 minute following treatment and was decreased to basal control levels, after 1 hour of treatment.

BPs transiently increased ERK 1/2 phosphorylation within 1 minute of treatment, returning to baseline by 5 minutes, as shown previously by Plotkin et al. (Plotkin et al., 1999). In this treatment group baseline levels were maintained for all subsequent time points investigated, up to 5 hours (data not shown). Addition of Dex to cells pre-treated for 1 hour with BPs resulted in a reduced activation of ERK 1/2 relative to samples treated with Dex alone (Fig. 6B). The MEK 1/2 protein inhibitor, UO126, blocked Dex and/or BP-induced ERK 1/2 activation in all cases.

In order to further characterize the role of ERK1/2 pathway in Dex-induced apoptosis, the presence of proteins lying both upstream (MEK1/2 and C-Raf) and downstream (p90rsk) of ERK1/2 protein, was investigated. Western blot analysis showed that MEK (Fig. 7A) and p90rsk (Fig. 7B) activation by Dex coincided with ERK1/2 activation, while pre-treatment with ALN slightly reduced phosphorylated levels of both proteins. In addition, UO126 prevented activation of p90rsk by Dex, but did not affect phosphorylated MEK1/2 protein, in accordance with previous reports (Favata et al., 1998). Levels of c-Raf remained constant and similar to vehicle levels, during all different treatments and time points investigated (Fig. 7C).

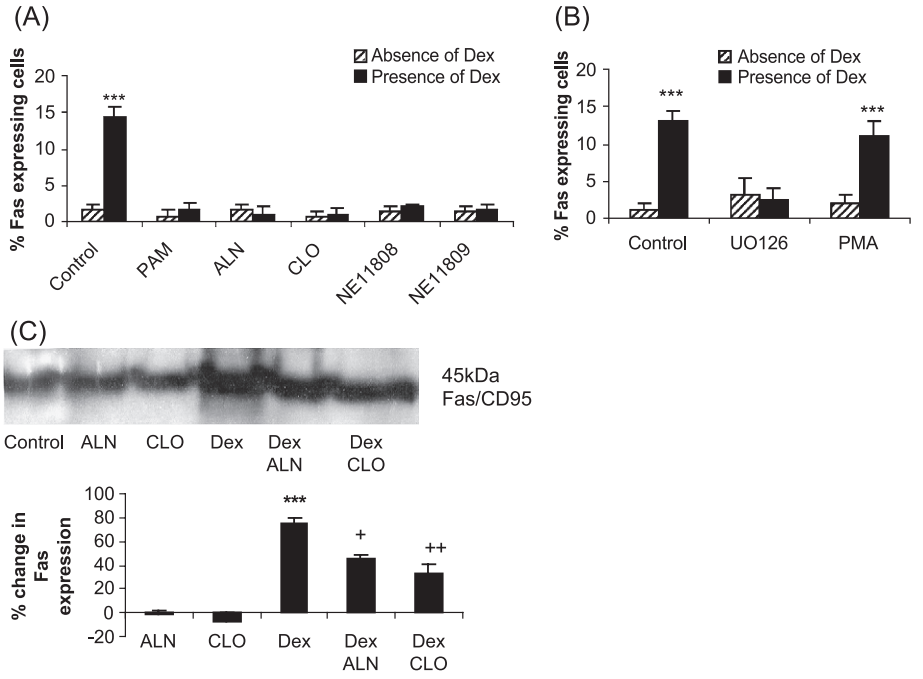

Fig. 8. BPs and UO126 suppress Dex-induced Fas expression. Cells were probed with an anti-Fas antibody and examined by fluorescence microscopy. (A) N- and non N-BPs reduce Dex-induced Fas expression as shown by immunocytochemistry and western blot analysis. Changes in band densitometry were quantified using NHI image analysis system and expressed as percentage change relative to vehicle sample ± S.D. from 3 independent blots (B) PMA alone did not increase Fas expression, compared to control. Graphs represent percentage of osteocytes, expressing Fas per number of cells ± S.D. (*** = $p < 0.0001$, compared to control, ++ = $p < 0.001$, + = $p < 0.05$ compared to Dex treatment).

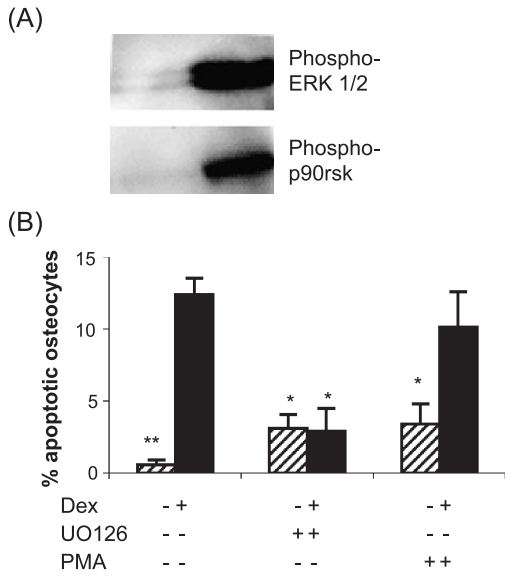

Fig. 9. PMA-induced ERK1/2 activation is not associated with osteocyte apoptosis. Cells treated with PMA followed by Dex were (A) subjected to western blot analysis to reveal pERK1/2 and p90rsk activation and (B) treated to reveal DNA breaks using the Nick Translation assay. PMA did not affect osteocyte apoptosis, compared to control. Graphs represent percentage of apoptotic osteocytes, per number of cells ± S.D. (*** = $p < 0.0001$, ** = $p < 0.001$, * = $p < 0.05$).

Suppression of Dex-induced Fas activation by MEK 1/2 inhibitor and BPs

The role of BPs and ERK 1/2 protein kinase in Dex-induced activation of Fas was investigated by immunocytochemistry studies and western blot analysis (Fig. 8). Previous studies have shown interaction between ERK and Fas protein pathways in the induction of apoptosis (Goillot et al., 1997). Pre-treatment of osteocytes with UO126 at 20 μM, prevented activation of Fas by Dex ($p=0.0001$, compared to Dex-treated samples). In a similar manner to UO126, pre-treatment of MLO-Y4 cells with both N- and non N-BPs at 10^{-8} M, reduced activation of Fas by Dex ($p=0.0001$).

PMA-induced ERK1/2 activation is not associated with osteocyte apoptosis

PMA did not induce osteocyte apoptosis (Fig. 8), despite demonstrating a clear increase in phosphorylated ERK in MLO-Y4 osteocytes as determined by western blotting (Fig. 9A). Furthermore, PMA when administered alone at concentrations of 2 ng/ml to 200 μg/ml for 1 to 5 hours did not increase Fas, while prior to Dex treatment PMA did not enhance Dex-induced Fas upregulation, in contrast to Dex treated cultures (Fig. 9B).

Discussion

Prolonged administration of glucocorticoids leads to bone loss and osteoporosis, through stimulation of resorption and induction of osteoblast and osteocyte apoptosis (Weinstein et al., 1998). This study has

attempted to investigate molecular pathways implicated in Dex-induced death of osteocytes, the mechanosensors and transducers in bone, and on molecules that might provide therapeutic approaches to combat this death. Dex induction of osteocyte apoptosis characterised by several classical apoptotic features, including chromatin and cytoplasmic condensation, DNA fragmentation, exposure of phosphatidylserine and formation of apoptotic bodies, was concentration- and time-dependent. In addition, apoptosis was caspase-dependent since inhibition of caspases −3 and −7 (Lee et al., 2000), which are responsible for chromatin condensation, DNA fragmentation and membrane blebbing, suppressed Dex-induced death.

Dex-induced apoptosis has been associated with the Fas/FasL apoptotic pathway in several different cell types, in relation to its action as an immunosuppressive agent (Schmidt et al., 2001). Fas receptor mRNA was detected in MLO-Y4 osteocytes both in response to Dex treatment and in untreated cultures. However, Dex treatment upregulated localisation of Fas protein on the osteocyte plasma membranes, in a time-dependent manner compared to untreated controls, indicating a possible association between Fas recruitment and the incidence of osteocyte apoptosis in the presence of Dex. Indeed, inhibition of caspase 8 using a peptide inhibitor (Martin et al., 1998), the upstream caspase participating in the Fas pathway, blocked Dex-induced apoptosis, further supporting a possible association between Dex and the Fas pathway in osteocyte apoptosis. However, we failed to detect expression of Fas Ligand in both Dex-treated and untreated cultures, suggesting that if indeed there is activation of the Fas-related pathway in the presence of Dex it is FasL independent. Similar phenomenon has been observed in tumour cell studies, showing that anticancer agents directly promote Fas receptor trimerisation and activation of the FADD/caspase 8 pathway, independently of FasL (Misceau et al., 1999).

BPs suppressed Dex-induced apoptosis in MLO-Y4 osteocytes after 5 hours treatment with Dex, in accordance to previous reports by Plotkin, et al. (Plotkin et al., 1999). Although we do not know the half-life of BPs in in vitro experimental conditions, the pharmacokinetics of these molecules in vivo, might allow them to suppress GC-induced apoptosis of osteocytes for longer periods of time, since they can be retained active in the skeleton until their release, following resorption of the multiple sites in which they were deposited.

Dex-induced apoptosis was prevented by both non N-BPs (CLO), which are metabolized into cytotoxic analogues of ATP and N-BPs (PAM and ALN), which inhibit prenylation through inhibition of FPP synthase (Rogers et al., 1999). The third generation heterocyclic-containing NE11808 was used as well as the structurally similar NE11809 which differs in terms of having a methyl group, which confers reduced inhibition of FPP synthase (Dunford et al., 2001). Both BPs were equally effective inhibitors of death, indicating that prevention of Dex-induced osteocyte apoptosis by BPs does not depend on inhibition of FPP synthase or on structure/activity relations of BP molecules.

ERK 1/2 is associated variously with the induction of proliferative and survival signals (Robinson and Cobb, 1997; Lai et al., 2001; Jamieson and Yamamoto, 2000; Tran et al., 2001). However, in contrast to the known pro-survival effects, ERK is also implicated in cisplatin-induced apoptosis in HeLa cells, brain injury during focal cerebral ischemia and activation-induced cell death of T cells (Wang et al., 2000; Alessandrini et al., 1999; Van den Brink et al., 1999).

Recently, Nishida, et al have reported that YM529, a new bisphosphonate, decreases phosphorylation of ERK1/2 during apoptosis of HL60 cells (Nishida et al., 2003). Nevertheless, Plotkin, et al observed an acute activation of ERK by BPs, which was sustained for 5 minutes during

the pre-treatment period (Plotkin et al., 1999), and suggested that activated ERK is involved in the protective effects of BPs on Dex-induced apoptosis. Our study has also shown activation of ERK by BPs, which peaked at 5 minutes of addition of BPs to osteocyte cultures. However, Plotkin, et al did not measure possible ERK production in response to Dex, as has been noted in other cell types (Jamieson and Yamamoto, 2000). Following on from both studies, our work has investigated the course of ERK activation in order to characterize the effect of Dex on the ERK pathway, during osteocyte apoptosis. In contrast to activation of ERK by BPs during the pre-treatment period, Dex transiently increased the amount of activated ERK1/2 in osteocytes, which remained elevated for the first hour of incubation, and was suggestive of a specific non-genomic effect, involving membrane-bound GC receptors, since activation of cytosolic receptors requires at least 30 minutes (Patschan et al., 2001). Inhibition of ERK by UO126 suppressed Dex-induced osteocyte apoptosis, both in the presence and absence of BPs, indicating that induction of death signals by Dex-activated ERK compared to non-damaging BP-activated ERK is due to either differences in the timing and duration of activation or to the generation of secondary factors by Dex, which render ERK pro- rather than anti-apoptotic. Furthermore, our data indicated a significant reduction in ERK phosphorylation induced by Dex, in the presence of BPs. Variance between the findings of Plotkin et al., in which ERK appears as an anti-apoptotic signal and our current conclusion regarding its positive role in apoptotic death, may be due to differences in experimental conditions between the two studies. Plotkin et al studied apoptosis in serum-replete conditions and ERK under serum free conditions, while we studied both apoptosis (the phenomenon) and ERK phosphorylation in these cells under identical serum-replete conditions. On the other hand, PMA treatment failed to increase the proportion of apoptotic osteocytes when added alone, and did not prevent or enhance the pro-apoptotic stimuli in the presence of Dex, indicating that activation of ERK through other pathways is not sufficient to induce osteocyte apoptosis.

Dex treatment also increased MEK and p90rsk activation at identical times to the activation of ERK, which was however reduced in cultures pre-treated with BPs, whereas levels of c-Raf were not altered compared to control levels, suggesting that Dex is acting downstream of c-Raf or through another isoform of Raf in the signalling pathway involving Raf, MEK and ERK. In contrast to ERK, p38 inhibition did not reduce osteocyte apoptosis, indicating specificity in the pro-apoptotic effects of MAPK family members.

In neuroblastoma cells transfection with activated MEK1 upregulated Fas activity (Goillot et al., 1997), while in T cells, during activation-induced cell death, transfection of a dominant negative MEK1 inhibited FasL expression (Van den Brink et al., 1999). In our study, inhibition of ERK in experiments using the Fas/CD95 antibody reduced the Dex-induced Fas activation, whereas upregulation of ERK by PMA did not affect Fas levels, indicating that production of Fas is closely associated to the pro-apoptotic signals induced by Dex. In addition, prevention of Dex-induced apoptosis by non N-BPs and N-BPs also decreased Fas expression, supporting the importance of Fas in the death response and pointing to the existence of an additional factor associated with Dex-treatment that might enable the co-operation between ERK and Fas pathways during the induction of osteocyte apoptosis. In conclusion, inhibition of Dex-induced osteocyte apoptosis by BPs was independent of the FPP synthase pathway associated with strongly anti-resorptive molecules, suggesting that BPs might in principal by modifying the structure or conformation of the R2 side chain, be independently applied to affect osteoclast activity or osteocyte survival, providing therapeutic approaches for various bone diseases induced by glucocorticoids.

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