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Ajulemic acid, a nonpsychoactive cannabinoid acid, suppresses osteoclastogenesis in mononuclear precursor cells and induces apoptosis in mature osteoclast-like cells.

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ABSTRACT

Ajulemic acid (AjA) is a synthetic analog of the tetrahydrocannabinol (THC) metabolite THC-11-oic acid. AjA reduces release of the inflammatory cytokine interleukin-1β (IL-1β) from peripheral blood and synovial fluid monocytes, and prevents bone degradation in a rat model of inflammatory arthritis. Because preosteoclasts are of monocyte lineage, we initiated these studies to understand the direct effects of AjA on both osteoclast differentiation and activity. In this paper, we demonstrate that AjA suppresses osteoclastogenesis *in vitro* in a concentration dependent manner.

INTRODUCTION

Osteoclasts, responsible for bone resorption, are large multinucleated cells formed by fusion of hematopoietic precursor cells of the monocyte/macrophage family. Osteoclasts usually have between 10 and 20 nuclei per cell but may have as many as 100 (Roodman, 1996). They are formed continuously throughout life under normal physiological conditions to maintain calcium homeostasis and to support normal bone remodeling. In response to hormones and mechanical stimuli, the mononuclear hematopoietic lineage cells fuse to form the differentiated osteoclast (Saltman, 2005). During osteoclast differentiation, signature phenotypic markers are expressed including tartrate-resistant acid phosphatase (TRAP), the calcitonin receptor, matrix

metalloproteinase 9 (MMP-9), and cathepsin K (Suda, 1999; Vaananen and Zhao, 2002; Boyle, 2003; Teitelbaum and Ross, 2003; Saltman, 2005).

The discovery that receptor activator of NF-kB ligand (RANKL) is the major physiological mediator of osteoclast differentiation enabled investigators to generate pure populations of osteoclasts *in vitro* from the mononuclear phagocyte precursor (Lacey, 1998; Yasuda, 1998). This major breakthrough led to a greater understanding of the mechanisms controlling osteoclast differentiation and activity under normal biological and pathological conditions (Boyle, 2003; Teitelbaum and Ross, 2003; Saltman, 2005). A murine macrophage cell line, RAW264.7, can differentiate readily into osteoclast-like cells in the presence of RANKL (Hsu, 1999). Known markers of osteoclasts are induced in the multinuclear TRAP-positive cells that form from the RAW264.7 cells, confirmation that these cells possess major characteristics of osteoclasts (Hotokezata, 2002).

RANK signaling pathways driven by RANKL, are controlled at several levels resulting in enhanced or reduced osteoclastogenesis. Activation of osteoclast surface receptors for IL-1, c-Fms proto-oncogene, tumor necrosis factor alpha (TNF-α), prostaglandin E_2 (PGE_2), and T-cell growth factor beta (TGF-β) potentiate osteoclastogenesis *in vitro*, and can stimulate bone resorption *in vivo* (Boyle, 2003).

The Cannabis plant has been a source of medicinal preparations since the earliest written records on pharmacobotany. A major obstacle to broad acceptance of cannabinoids as therapeutic agents is their potent psychoactive effects. A class of cannabinoids, the carboxyl tetrahydrocannabinols, which are metabolites of tetrahydrocannabinol (THC), shows promise as therapeutic agents that are free of cannabimimetic central nervous system activity (Burstein, 1972). These compounds, called cannabinoid acids, include all the carboxylic acid metabolites of

the cannabinoids and their synthetic analogs. One analog, 1'1'-dimethylheptyl-THC-11-oic acid, termed ajulemic acid (AjA), is a potent antiinflammatory and analgesic agent in several animal models (Dajani, 1999). In addition, AjA is not psychoactive in mice. In fact, AjA suppresses THC-induced catalepsy in mice (Burstein, 1999). AjA, given to rats orally, reduces significantly the severity of adjuvant-induced polyarthritis in rats. Histomorphological evaluation of the joints suggested that synovial inflammation occurred in treated animals, but that it did not progress to cartilage degradation, bone erosion, and distortion of joint architecture, as was observed in rats given placebo (Zurier, 1998). Results of experiments presented in this paper indicate that AjA suppresses osteoclastogenesis *in vitro*.

Many adult skeletal diseases are due to excess osteoclastic activity, leading to an imbalance in bone remodeling which favors resorption (Rodan, 2000). These include osteoporosis, periodontal disease, rheumatoid arthritis, multiple myeloma, and metastatic cancers (Boyle, 2003). For individuals with osteoporosis, bone fractures can represent life-threatening events, and today in excess of 70 million people worldwide are at risk (Boyle, 2003). In an initial examination of the effects of AjA on osteoclasts, we observed that addition to mononuclear precursors suppressed osteoclastogenesis and also reduced cell number. In an investigation of the observed reduced cell number, we discovered that AjA induced apoptosis of osteoclasts. Apoptosis is recognized as a mode of cell death in which cells are deleted in the midst of living tissue (Kerr, 1972). Apoptotic cells express characteristic features such as cell shrinkage, nuclear condensation, loss of membrane microvilli, and surface blebbing (Wyllie, 1984). Fragments of apoptotic cells are digested by resident phagocytes without influx of inflammatory cells (Polunovsky, 1993). Thus, in contrast to cell death by necrosis, apoptosis is regulated and has been termed "programmed cell death" (Cohen, 1993). It is possible that the effects of AjA on

osteoclastogenesis and on osteoclast cell number will have a protective effect in diseases such as osteoporosis and rheumatoid arthritis characterized by excess osteoclast activity.

MATERIALS AND METHODS

AjA was obtained from Organix (Woburn, MA). Its purity was monitored on high-pressure liquid chromatography by comparison with material synthesized previously (Burstein, 1992). The sample was 97% chemically pure, and was >99% chirally pure in the enatiomer.

Establishment of Osteoclast Cultures. RAW264.7 cells were thawed and transferred to100mm plates (Corning, Corning, NY), and cultured 6-7 days in DMEM medium supplemented with 10% FBS, 2mM L-glutamine and 1% (v/v) penicillin-streptomycin (R&D Systems, Minneapolis, MN). To induce RAW264.7 cells to differentiate into osteoclasts, bacterially-produced recombinant RANKL (5ng/ml) was added. RAW264.7 cells were plated at the density of $2X10^5$/plate when no RANKL was added, and $4X10^5$/plate when RANKL was added.

Osteoclast staining. Osteoclast formation was monitored cytochemically by staining for tartrate-resistant acid phosphatase **(**TRAP). TRAP is regarded as an osteoclast marker and is mostly characterized by its biochemical property, i.e., through its acid phosphatase function, which cannot block tartrate (Filgueira, 2004). A histochemical method, commercially available as a kit by Sigma Diagnostics (St Louis, MO), is widely used and has proved to be the standard method for specific detection of osteoclasts. After TRAP staining, the numbers of osteoclasts (with ≥3 nuclei) were quantified by counting in 3 different fields.

RNA Isolation and real-time PCR. Total cellular RNA was isolated using Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA) as per manufacturer's directions and

resuspended in DEPC-treated water. For RT-PCR, RNA was treated with DNase I and purified using a DNA-freeRNAkit (Zymo Research, Orange, CA). cDNA was synthesized using the Invitrogen SuperScript First Strand Synthesis System with oligo dT primers. GAPDH, BAX, BCL2, and Histone H4b mRNA were amplified using iTaq SYBR green supermix (Bio-Rad Laboratories, Hercules, CA) and 2.5 pmoles of each primer (Applied Biosystems, Foster City, CA). Conditions for all PCR reactions were as follows: 50°C for 2 min, 95°Cfor 10 min, followed by 40 cycles of 95°C for 15 sec, and 60°C for 1 min. Primers and probes for the TRAP, RANK receptor, cathepsin K, and MMP9 genes were all Assays-on-Demand products from Applied Biosystems and reactions were set up according to the manufacturer's directions. Real time PCR was performed in triplicate in 25 ml reactions on the ABI Prism 7000 Sequence Detection System. (Saltman, 2005) The relative level of each mRNA was determined using the comparative CT method for relative quantitation using GAPDH as an endogenous reference (Livak and Schmittgen, 2001).

Cell Growth. RAW264.7 cells ($1X10^6$) were plated and allowed to settle for 2 days. AjA was added to the media on the third day. Cells were scraped, pelleted, and resuspended in 1mL of PBS on each of the three days of AjA treatment. Cells were then counted in a standard hemocytometer.

Annexin V Staining for Apoptosis. Phosphatidyl serine (PS) is located in the inner leaflet of the plasma membrane. Early in the apoptotic process, reorganization of the phospholipid bilayer leads to the exposure of PS on the cell surface. The anticoagulant annexin-V binds with high affinity to PS in the presence of calcium (van Engeland, 1997).

RAW264.7 cells ($1X10^6$) were plated and allowed to settle for 2 days. RANKL and AjA were added to the fresh media on the third day. Media was replaced and fresh RANKL and AjA

were added on the fifth day. Cells were processed on each of the three days of RANKL and AjA treatment. Cells were scraped and the cell number was adjusted to $1X10^6$ cells/ml.
Cells were prepared using the RAPID Annexin V Binding Protocol from the Annexin V FITC Apoptosis Detection Kit (Calbiochem, La Jolla, CA). Annexin V binding was measured by flow cytometry.

Caspase-8 and Caspase-3 Activities. Caspase-8 and caspase-3 activities were quantified using a colorimetric assay in which caspases recognize the amino acid sequence IETD (Chemicon International, Temecula, CA). RAW264.7 cells were prepared as described in "Materials and Methods" with the addition of AjA first (24hr), washout, and then RANKL (3 days). A pellet of $5x10^5$ cells was resuspended in 1x Cell Lysis Buffer and then treated according to the manufacturer's protocol. Cleavage of the labeled substrate IETD-*p*NA by caspase-8 released the chromophore *p*-nitroaniline (*p*-NA). The free *p*NA light absorbance was quantified using a microplate reader at 405nm. Comparison of the absorbance of *p*NA from the apoptotic samples with an untreated control allowed the determination of the fold increase in caspase-8 and caspase-3 activity.

RESULTS

Suppression of Osteoclastogenesis by Ajulemic Acid.

AjA reduces release of the inflammatory cytokine interleukin-1β (IL-1β) from peripheral blood and synovial fluid monocytes, and prevents bone degradation in a rat model of inflammatory arthritis (Zurier, 2003; Zurier, 1998). Because preosteoclasts are in the monocyte lineage, we initiated these studies to understand the direct effects of AjA on both osteoclast differentiation and activity. Therefore, our experimental approaches examined the effects of AjA

on the RANKL dependent induction of mononuclear cells to fuse and differentiate into osteoclasts, and the effects of AjA on the activity of mature osteoclasts. We first established conditions for introducing AjA and RANKL into the RAW264.7 cell cultures. AjA added simultaneously with RANKL to induce RAW264.7 cells results in suppression by AjA of osteoclastogenesis (osteoclasts are not present at the initial time of treatment) (Fig 1). Addition of RANKL for 3 days before addition of AjA changes the condition to one in which AjA affects osteoclasts that are already formed. Addition of AjA first, washing it out, and then treating RAW264.7 cells with RANKL indicates whether the cells retain a "memory" of their exposure to AjA.

First, we examined the simultaneous addition of AjA and RANKL to mononuclear RAW264.7 cells and monitored osteoclastogenesis by tartrate-resistant acid phosphatase (TRAP) staining, osteoclast number, and expression of osteoclast marker gene mRNA levels. In a series of three experiments, it was found that when RAW264.7 cells were treated with RANKL and AjA together (day 0) for three days, osteoclastogenesis was consistently supressed in a dose dependent manner (Fig 1A). Low doses of AjA (0, 0.01, 0.1, and 1 μM) did not impair osteoclastogenesis substantially, whereas doses of AjA equal to or higher than 15 μM suppressed osteoclastogenesis significantly (Fig 1B). Osteoclast counts were performed by light microscopy in three different fields. We observed that osteoclasts (>3 nuclei) formed after exposure of cells to 15μM AjA all were smaller in size, but had fewer nuclei per cell compared to cells treated with 1μM AjA. These observations suggest that AjA impaired the ability of monocytes to fuse. In all three experiments osteoclastogenesis, as measured by TRAP staining and by direct counting, was reduced significantly by 20 μM or more of AjA.

To further evaluate the effect of AjA on osteoclastogenesis, real time RT-PCR was used to assess relative mRNA levels of the osteoclast markers cathepsin K, an early marker, and TRAP, a marker of fully mature osteoclasts. We found that 30 μM AjA, but not the 15μM dose, reduced relative mRNA levels of RAW264.7 cells treated with AjA and RANKL simultaneously for three days (Fig 2). In a series of 2 experiments, 30 μM AjA decreased TRAP mRNA 72.2%$\pm$5.1%, p=0.002 (mean$\pm$s.d., p vs control) and decreased cathepsin K mRNA 43.2%$\pm$49%, p=0.34 (mean$\pm$s.d., p vs control). Unexpectedly, the 15 μM dose resulted in a 3 fold increase in cathepsin K (Fig 2B) and a greater than 2 fold increase in TRAP (Figure 2A). To understand this finding we examined expression of the receptor activator of NF-κB (RANK) receptor to determine if the cells are hyper responsive to RANKL in the presence of AjA. As reported by others (Hsu, 1999), we found that RANK expression was the same in RAW264.7 cells in the absence or in the presence of RANKL. However, a dose related increase in RANK does occur in the presence of 15 and 30 μM AjA. These observations indicate that AjA induces RANK expression in both monocytes and osteoclasts to the same extent and is highly dose related, suggesting a stimulation of osteoclast activity. The significant induction of the two markers reflecting osteoclast activity, TRAP and cathepsin K at 15 μM are consistent with stimulation of the RANK receptor pathway. However at the 30 μM dose a significant 75% reduction in TRAP and cathepsin K were found, consistent with the fewer number of osteoclasts at this dose.

Effect of ajulemic acid on RAW264.7 cell growth.

In an effort to determine whether AjA suppression of osteoclastogenesis was due to an effect on the mononuclear cells or on the differentiated osteoclasts, we first investigated the effect of AjA on RAW264.7 cell growth. RAW264.7 cells were plated at 50% below normal density, allowed to adhere for 48 hours, and AjA added at 2 doses. The cells exhibited log phase

growth from day 1 to day 3 (Fig 3A). AjA reduced cell numbers in a dose-dependent manner on day 3. Thus, cell growth was reduced 21.9% at 15 μM and 46.1%, p=0.006 vs untreated controls at 30 μM respectively (Fig 3A). We next treated RAW264.7 cells with AjA for only 24 hr, before harvest on day 3 (Fig 3B). Cell numbers were not affected significantly (Fig 3B). Thus, AjA does not appear toxic to mononuclear cells. Our results indicate that growth of RAW264.7 cells is diminished by AjA before confluency (day 2; Fig 3A), thereby reducing the number of precursors available for osteoclast formation. These results are consistent with TRAP staining experiments described above in which fewer mononuclear cells and osteoclasts are present in AjA treated RAW264.7 cells in differentiating cultures (see Fig 1).

Apoptosis caused by Ajulemic Acid.

The results presented in Figure 3 are from experiments with mononuclear RAW264.7 cells. To determine whether AjA acts on the osteoclasts after RANKL induced RAW264.7 differentiation, we tested the effects of AjA under two conditions. First, we formed osteoclasts with RANKL before addition of AjA. RAW264.7 cells were treated with RANKL for 3 days, then with 30 μM AjA during the final 24 hours of exposure to RANKL when osteoclasts were present (Fig 4). TRAP staining was used to quantify the number of osteoclasts present on day 3. AjA (15-30 μM) prevented further osteoclastogenesis (Fig 4A) and reduced the number of osteoclasts significantly (Fig 4B). This result suggests that AjA induced apoptosis of differentiated osteoclasts.

To further address the possibility that AjA promoted apoptosis of both mononuclear cells and osteoclasts propidium iodide labeled cells and annexin V staining was assessed by FLOW cytometry (Fig 5). In normal viable cells, phosphatidylserine (PS) is located on the cytoplasmic surface of the cell membrane. Upon induction of apoptosis, rapid alterations in the organization

of phospholipids occurs in most cell types, leading to exposure of PS on the cell surface. Detection *in vitro* of externalized PS can be achieved through interaction with the anticoagulant annexin V. In the presence of calcium, rapid high affinity binding of annexin V to PS occurs. PS translocation to the cell surface precedes nuclear breakdown, DNA fragmentation, and the appearance of most apoptosis-associated molecules, making annexin V binding a marker of early-stage apoptosis. (van Engeland, 1997). For these studies RAW264.7 cells were treated or not with RANKL and with or without AjA on day 0 for 3 days. Both early (Fig 5A) and late (Fig 5B) stage apoptotic cells were increased each day. In the absence of AjA, RAW264.7 cells stimulated with RANKL (thus forming osteoclasts) exhibited an initial increase in apoptosis, indicating that apoptosis was induced in developing osteoclasts. Also, there was an increase in apoptosis at 24 hours in cultures treated with AjA and RANKL, which indicates an increase in apoptosis of RAW264.7 cells.

During apoptosis, the cysteine proteases (caspases) are activated (Nagata, 1997). Addition of AjA to formed osteoclasts for 24 hours increased caspase 3 and caspase 8 activities, further indicating induction of osteoclast apoptosis by AjA (Figure 6).

Washout studies.

To determine whether the continuous presence of AjA in the RANKL stimulated RAW264.7 cell cultures is required for the suppressive activity, washout studies were done in which RAW264.7 cells were exposed to AjA for 2 or 24 hours, then washed extensively, and incubated with RANKL for 3 days. The results (Fig 7) showed that osteoclast formation was reduced even when AjA had been washed out of the system. Inclusions were noted in some osteoclasts, again suggesting induction of apoptosis by AjA. Indeed, AjA increased caspase 8

activity (Fig 8). However, in washout studies with pure cultures of RAW264.7 cells, AjA did not induce inclusions or caspase 8 activity (not shown).

DISCUSSION

In this paper, we demonstrate that the synthetic nonpsychoactive cannabinoid ajulemic acid suppresses osteoclastogenesis *in vitro* in a concentration dependent manner. We observed (direct cell counts, histochemical staining, mRNA levels for osteoclast markers) that addition of AjA to RANKL stimulated precursor cells (RAW264.5 mouse macrophages) reduces osteoclast formation. These observations did not distinguish between an effect of AjA on the RAW264.7 cells or on the mature osteoclasts. Therefore, under the influence of RANKL, RAW264.7 cells were allowed to differentiate into osteoclasts before exposure to AjA. Results of these experiments indicate that AjA acts directly on osteoclasts to reduce their number. Inclusions observed in these cells suggested that the osteoclasts were undergoing programmed cell death. Assessment of apoptosis by FLOW cytometry (annexin V and propidium iodide) and measurement of caspases 3 and 8 confirmed that process. Although AjA is not toxic to RAW264.7 cells, it does induce apoptosis in these osteoclast precursors when they are exposed to RANKL. Thus, apoptosis of developing osteoclasts and of fully formed osteoclasts explains, at least in part, the capacity of AjA to suppress osteoclastogenesis *in vitro*. Moreover, washout studies indicate that continuous presence of AjA in not necessary for the anti-osteoclastogenesis effect.

The precise manner by which AjA signals the precursor cells or the osteoclasts is not clear. AjA binds only weakly to CB2, the predominant cannabinoid receptor on macrophages (Rhee, 1997). Although we have shown (Liu, 2003) that AjA binds to and activates the nuclear

receptor and transcription factor PPARγ, we have also observed (Johnson, 2007) that AjA suppresses production of several matrix metalloproteinases in a PPARγ independent manner.

Although osteoporosis is likely caused by complex interactions among local and systemic regulators of bone cell function, it is clear that excessive bone resorption resulting in loss of bone mass and strength is central to the process (Raisz, 2005). Recent experimental evidence indicates that activation of osteoclasts is also central to the pathogenesis of bone resorption in joints of patients with RA (Gravallese, 2002). It is clear that cytokines such as interleukin-1 (IL-1) and IL-6 facilitate not only inflammation and immune responses, but bone resorption as well. Both IL-1 and IL-6 stimulate osteoclastogenesis (Wei, 2005; Kotakeetal, 1996). It is of interest then that AjA suppresses production of IL-1β by human peripheral blood and synovial fluid monocytes (Zurier, 2002), and of IL-6 by human monocyte derived macrophages (Parker, unpublished).

Endocannibinoids are endogenous compounds capable of binding to and functionally activating the two known cannabinoid receptors, CB1 and CB2 (Dimarzo, 2002). Recent observations suggest that endocannibinoids also interact with non-CB1, non-CB2 G protein coupled receptors and several ion channels (Dimarzo, 2002). The endocannabinoid system regulates bone mass in mice (Ofek, 2006; Karsek, 2004; Idris, 2005). CB2 deficient mice exhibit markedly accelerated age-related trabecular bone loss reminiscent of human osteoporosis (Ofek, 2006). In addition, a CB2-specific agonist enhances endocortical osteoblast number and activity, and impairs trabecular osteoclastogenesis (Ofek, 2006). In this context, our findings presented here that AjA suppresses osteoclastogenesis *in vitro*, and our observation that administration of AjA *in vivo* prevents bone erosion in adjuvant induced arthritis in rats (Zurier, 1998), encourage its further use in animal models, and suggest that AjA may prove to be safe, effective treatment

for osteoporosis and rheumatoid arthritis, conditions characterized by excessive osteoclast activity.

Now that psychoactivity can be separated from the medicinal properties of *Cannabis* products, and pure constituents are available or can be isolated from the plant, it is important to understand which constituents or combination of constituents of marijuana are responsible for the biological and pharmacological activities of the whole plant. Such knowledge, and synthesis of analogs of plant constituents, might lead to development of new, more benign treatment for diseases associated with inflammation and bone loss.

FIGURES AND LEGENDS

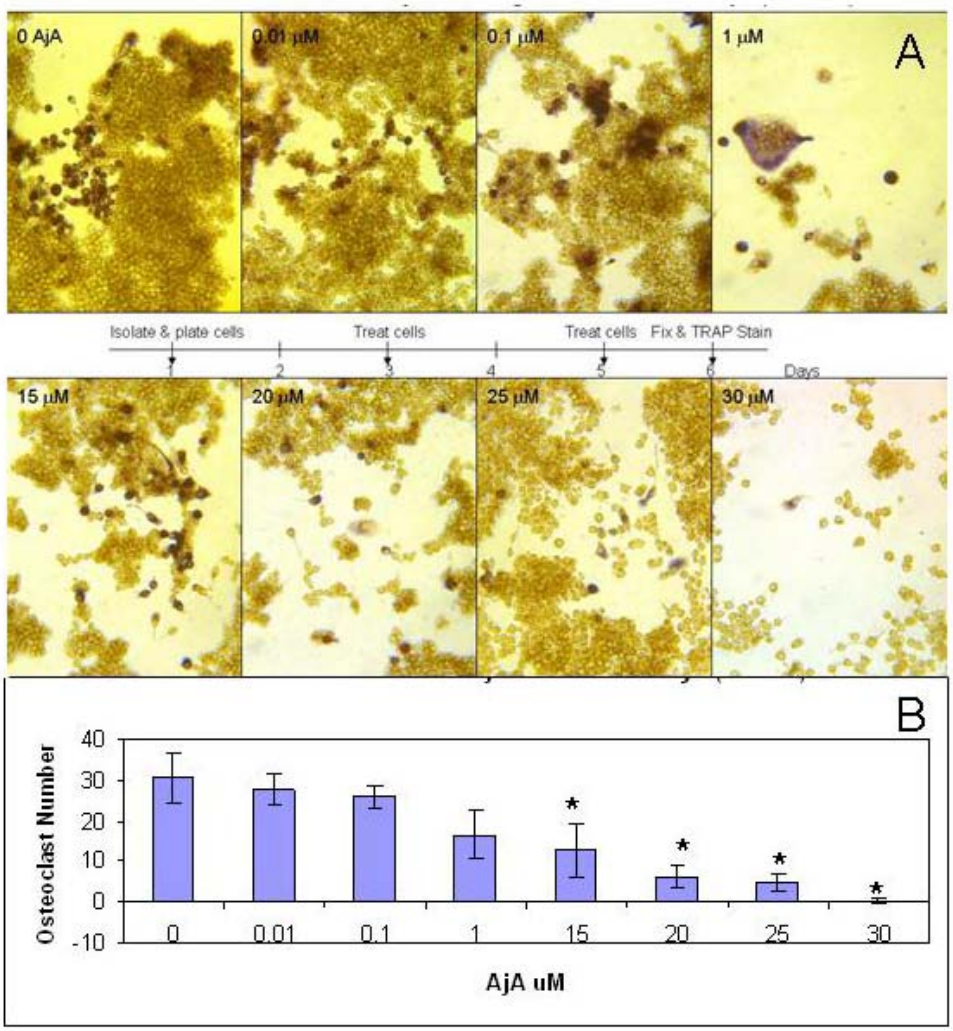


Figure 1 AjA suppresses osteoclastogenesis in a dose dependent manner. RANKL-treated cells were stained for TRAP and imaged A) One of three representative experiments shows cytochemical detection of TRAP decreased beginning from 15 μM dose. B) Counts by light microscope of osteoclast-like cells with 3 or more nuclei. At 15 μM, osteoclast-like cells have fewer nuclei, but were still present. Hence statistical significance was achieved beginning at 20 μM. Values shown are means $\pm$ sd of triplicate fields. * = $p<0.05$ vs. untreated controls.

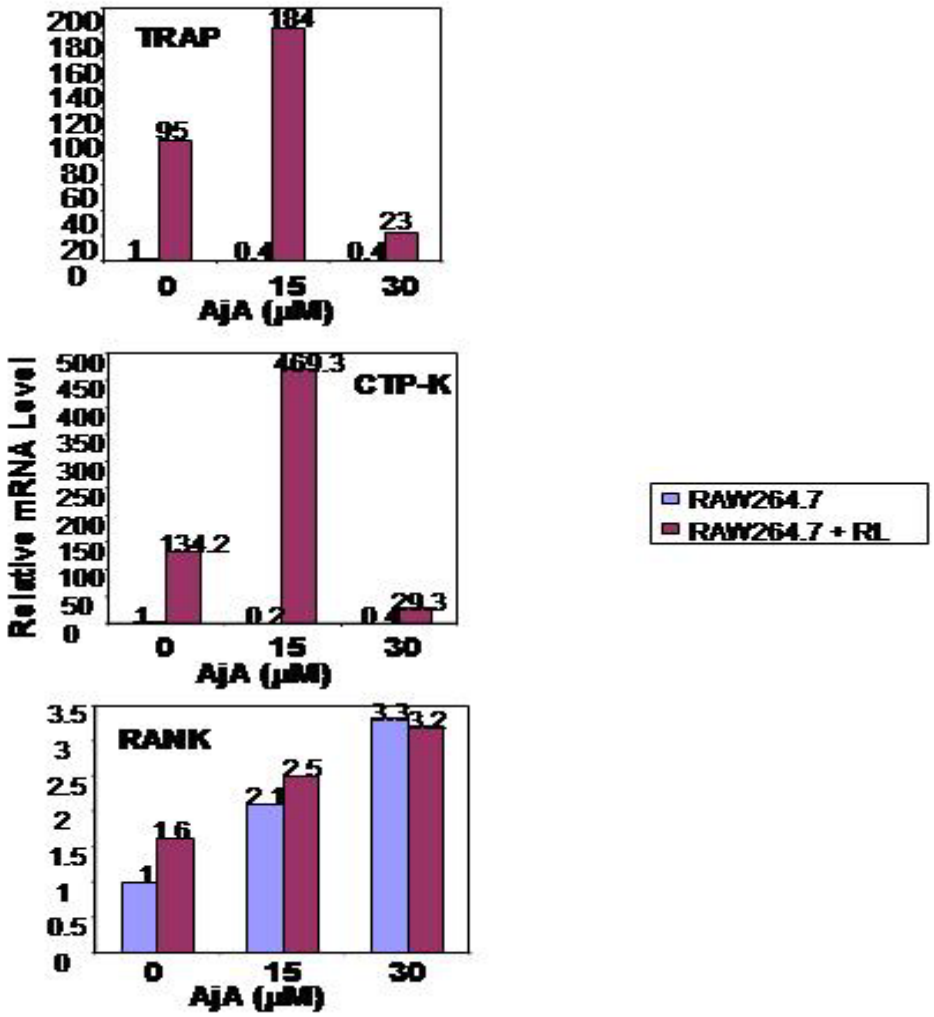
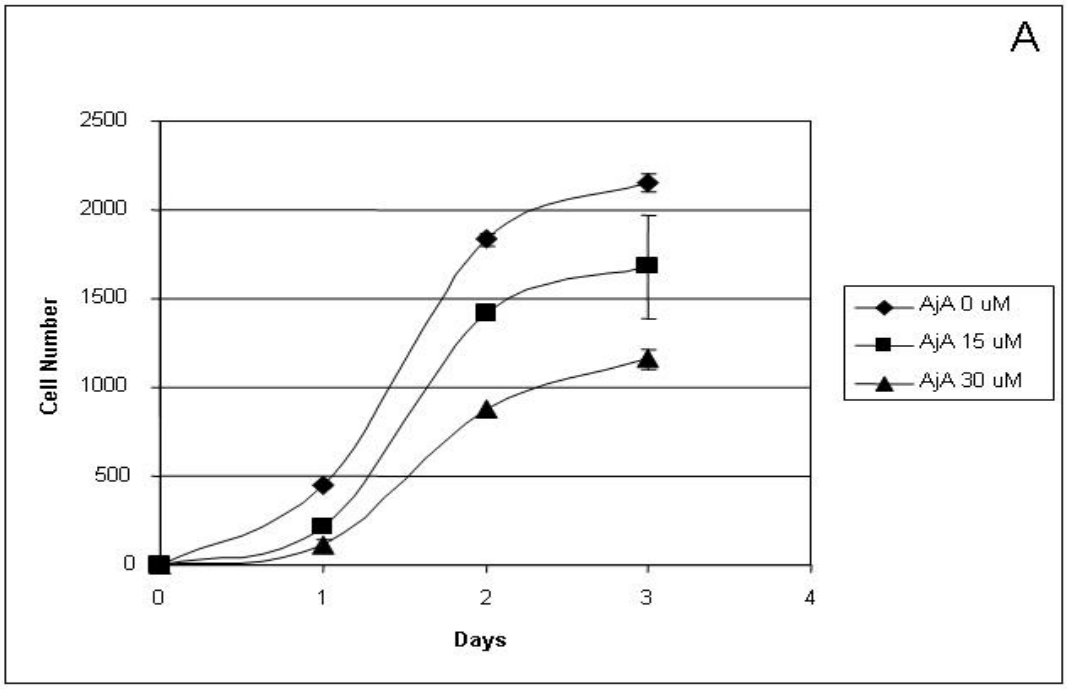


Figure 2 AjA suppresses osteoclastogenesis as shown by mRNA levels. Quantitative real time PCR of osteoclast phenotypic gene markers. Cells were cultured for 3 days in the absence (purple bar) or presence (magenta bar) of RANKL as described in "Materials and Methods." Total RNA was extracted, reverse transcribed, and subjected to real time PCR analysis using primers for known osteoclast markers. Values are normalized for GAPDH expression and expressed in arbitrary units.



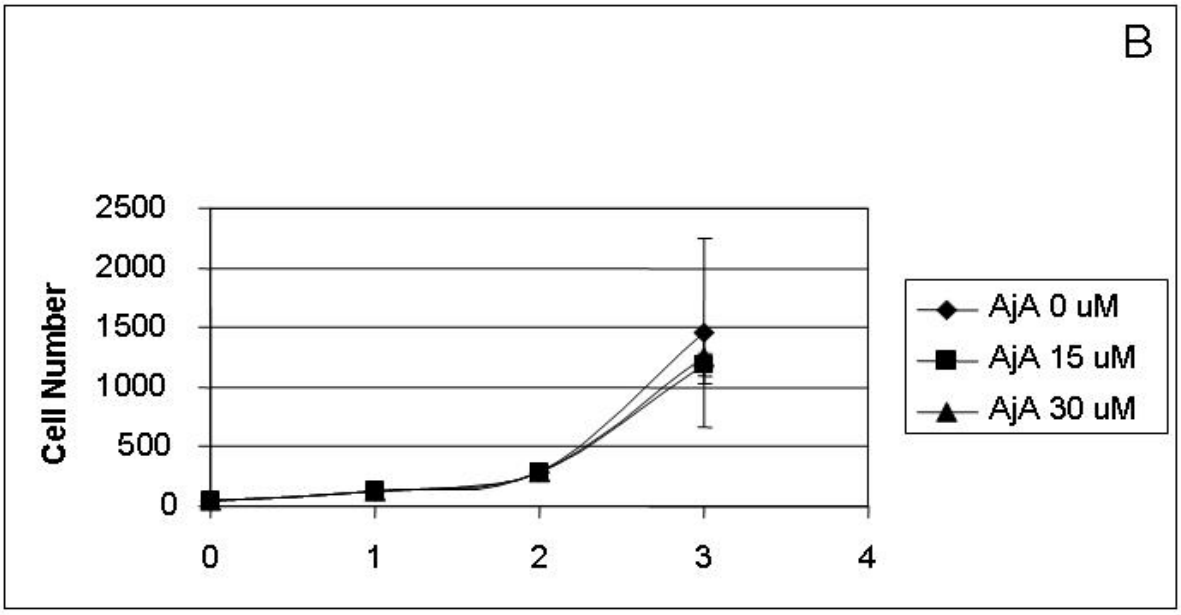


Figure 3 A AjA treatment of 3 days effects RAW264.7 cell growth. Values shown are mean $\pm$ sd of triplicate determinations. **B** AjA treatment of 24 hr does not effect RAW264.7 cell growth. Values shown mean $\pm$ sd of triplicate determinations.

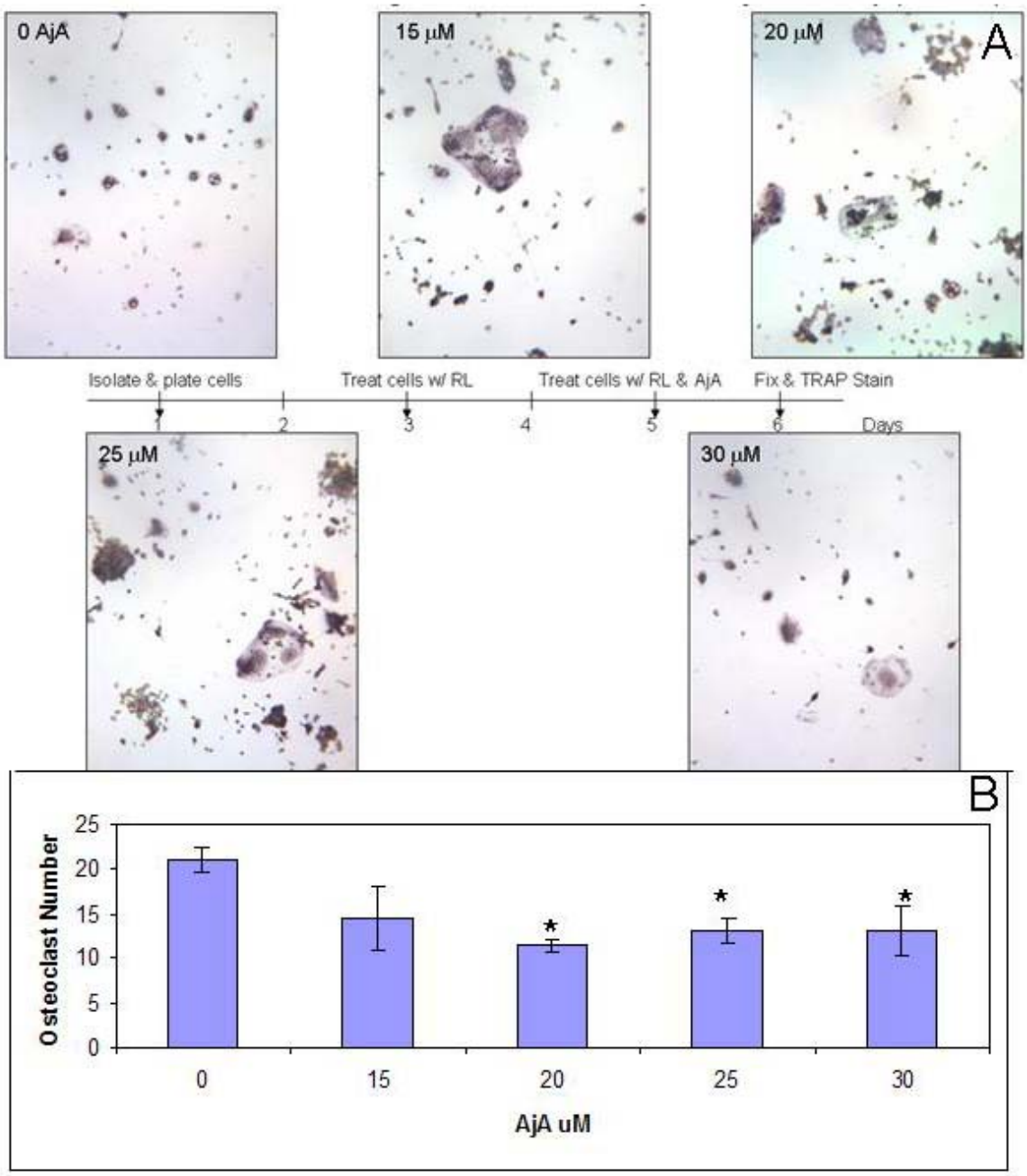


Figure 4 AjA suppresses osteoclastogenesis. Cytochemical detection of TRAP shown in (A) and osteoclast numbers shown in (B). The values shown are the mean + sd of triplicate fields. RANKL-treated cells were stained for TRAP and imaged.

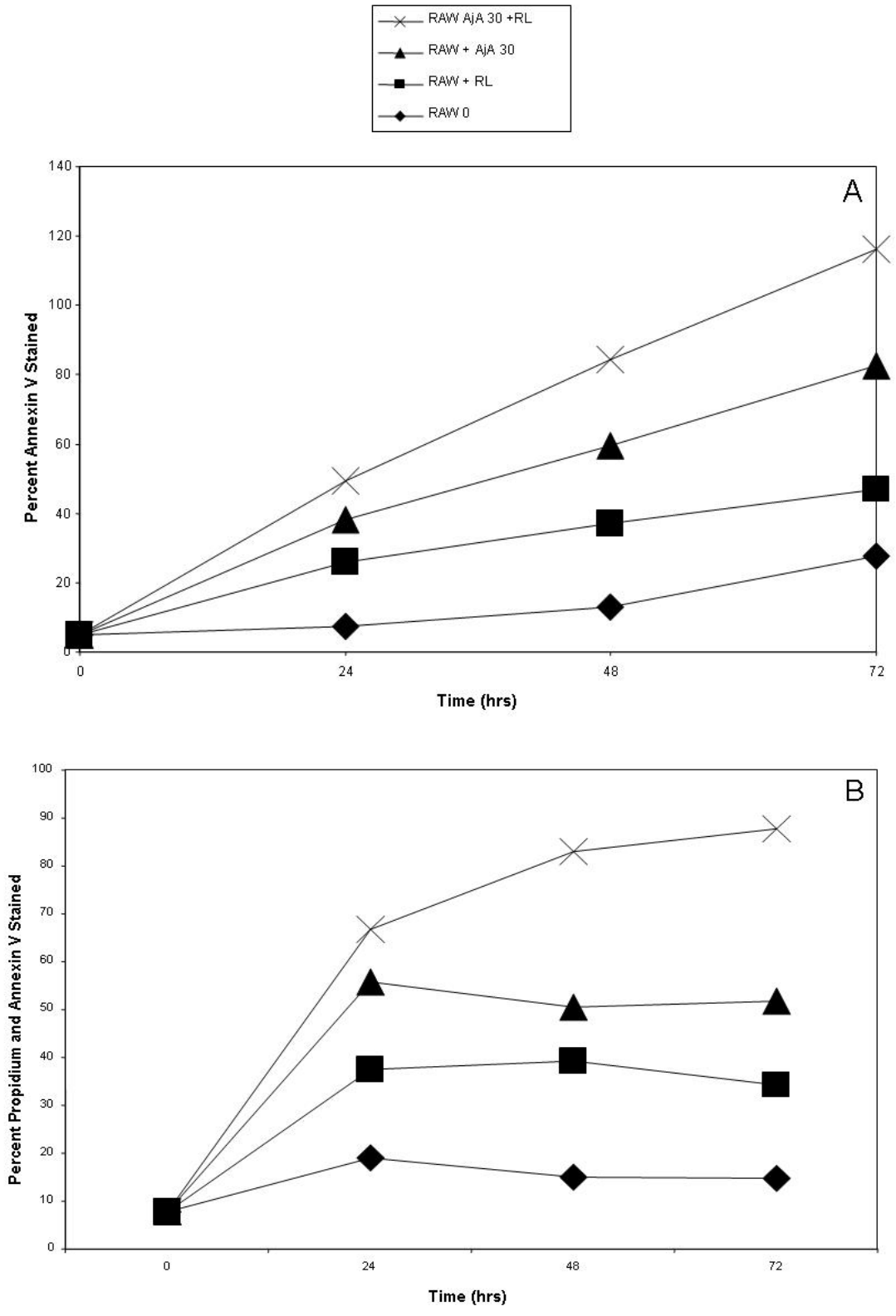


Figure 5 Apoptosis of RAW264.7 cells and osteoclasts: Annexin V and Propidium Iodide staining. Cells were isolated and prepared as described under "Materials and Methods". Apoptosis was analyzed by flow cytometry of cells stained with FITC-labeled Annexin V. Compared with control (RAW+RL), AjA increased the binding of Annexin V.

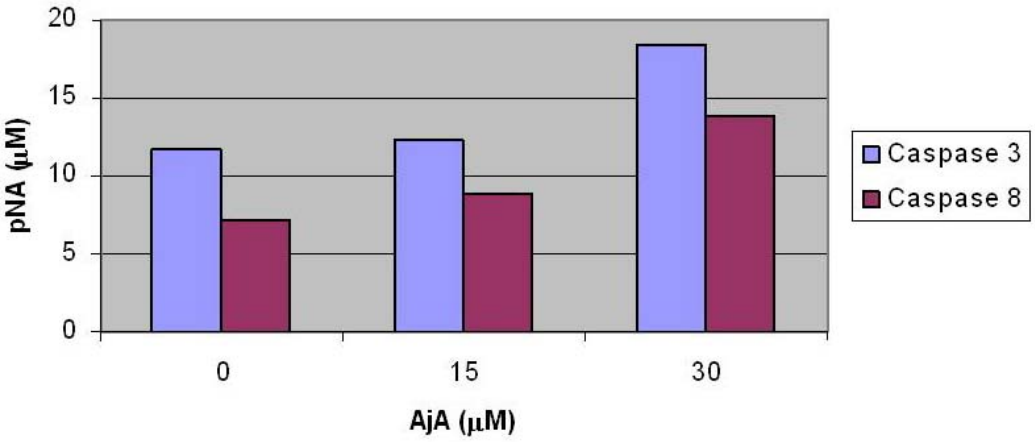


Figure 6 Apoptosis of osteoclasts: Caspase 3 and 8 activity. Osteoclasts incubated with AjA for 24 hours. Caspase-8 activity quantified by a colormetric assay as described in "Materials and Methods".

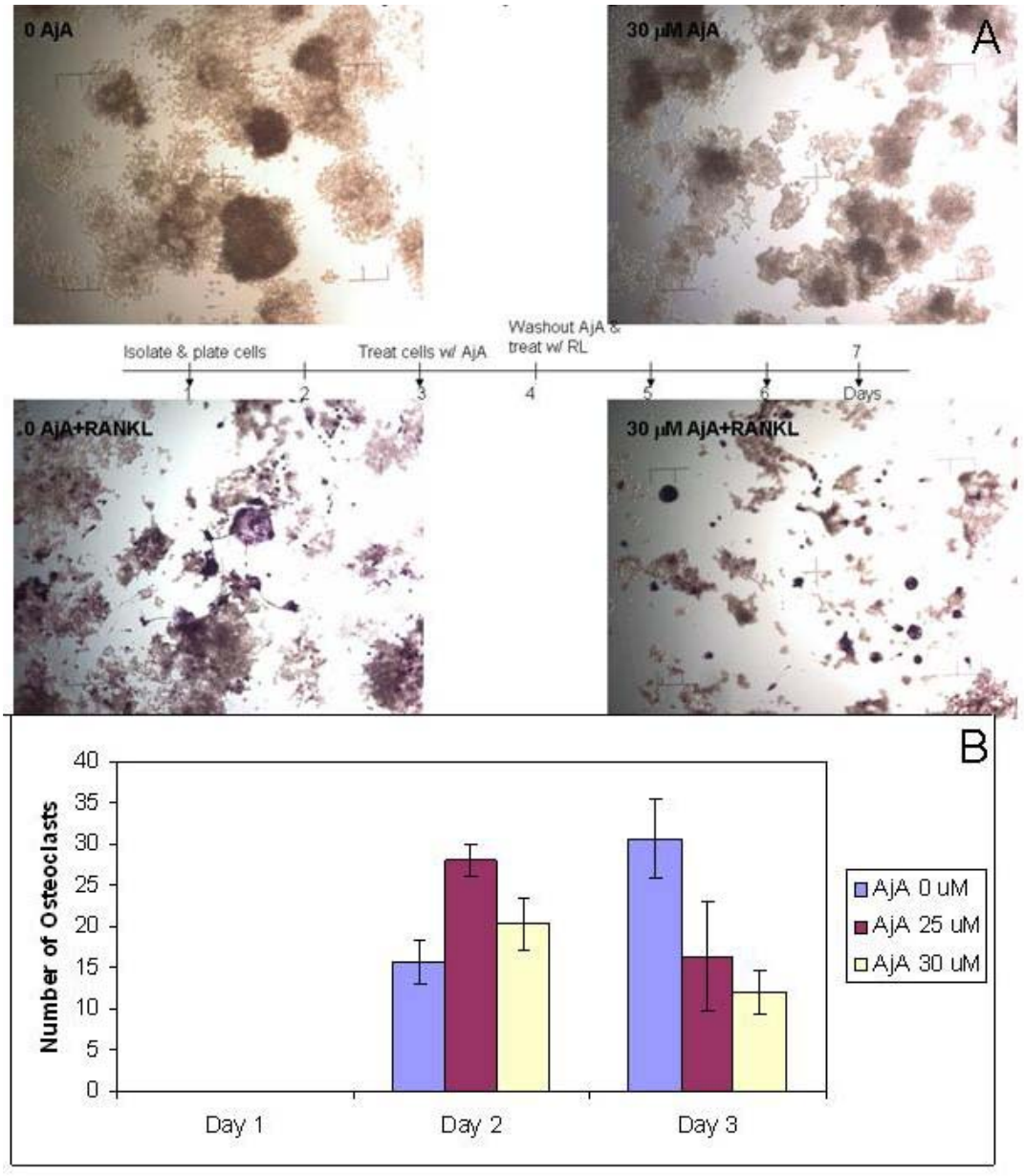


Figure 7 AjA suppresses osteoclastogenesis. Cytochemical detection of TRAP shown in (A) and osteoclast numbers shown in (B). The values shown are the mean ± standard deviation of triplicate fields. RANKL-treated cells were stained for TRAP and imaged.

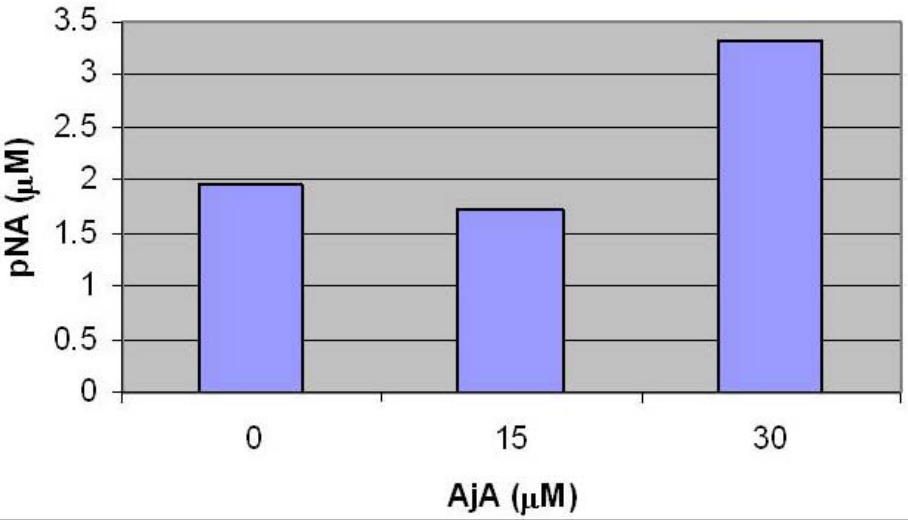


Figure 8 Apoptosis of RAW264.7 cells and osteoclasts: Caspase 8 activity. RAW264.7 cells incubated with AjA for 1 day and RANKL for 3 days (washout study). Caspase-8 activity quantified by a colormetric assay as described in "Materials and Methods".

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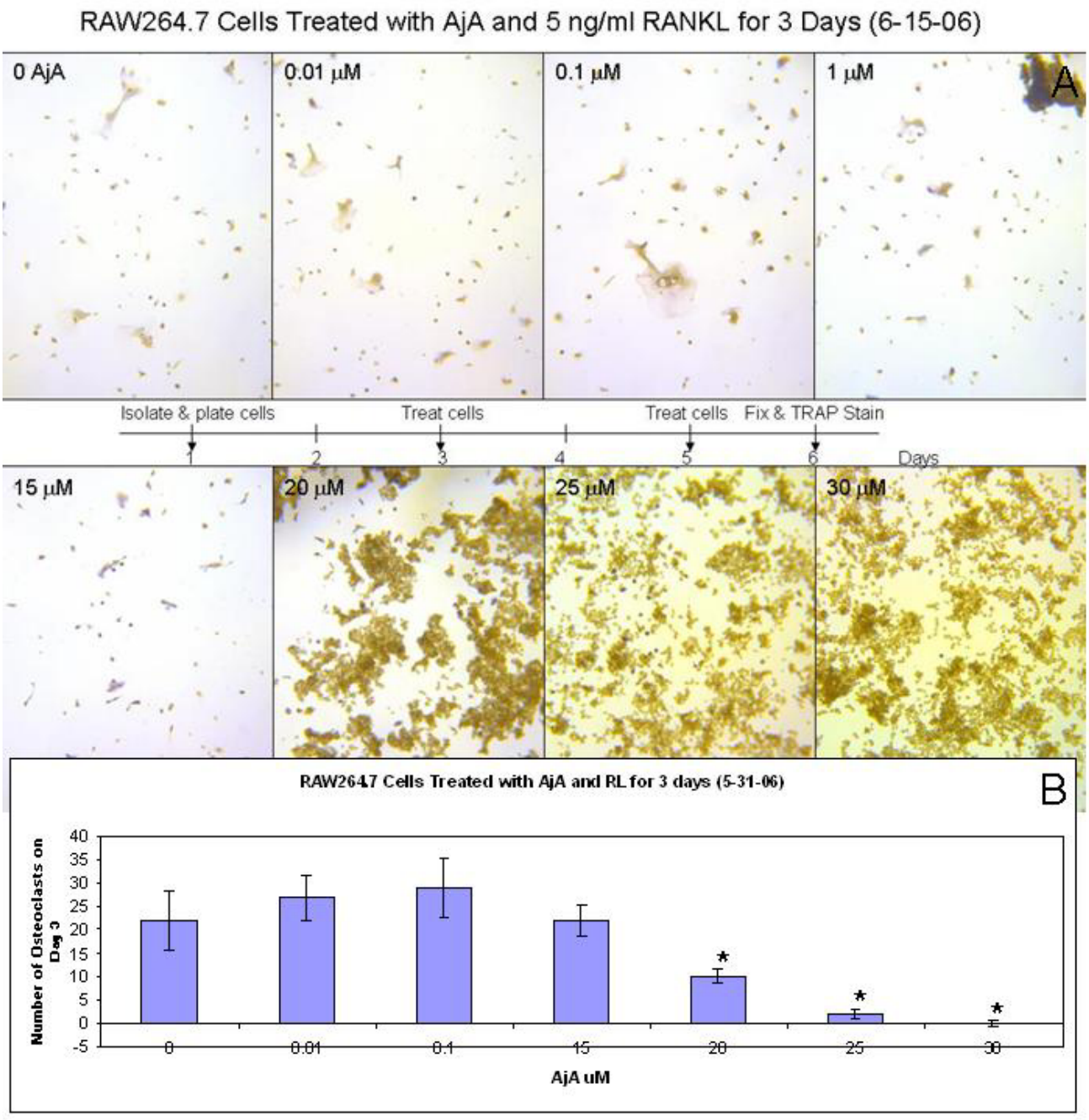
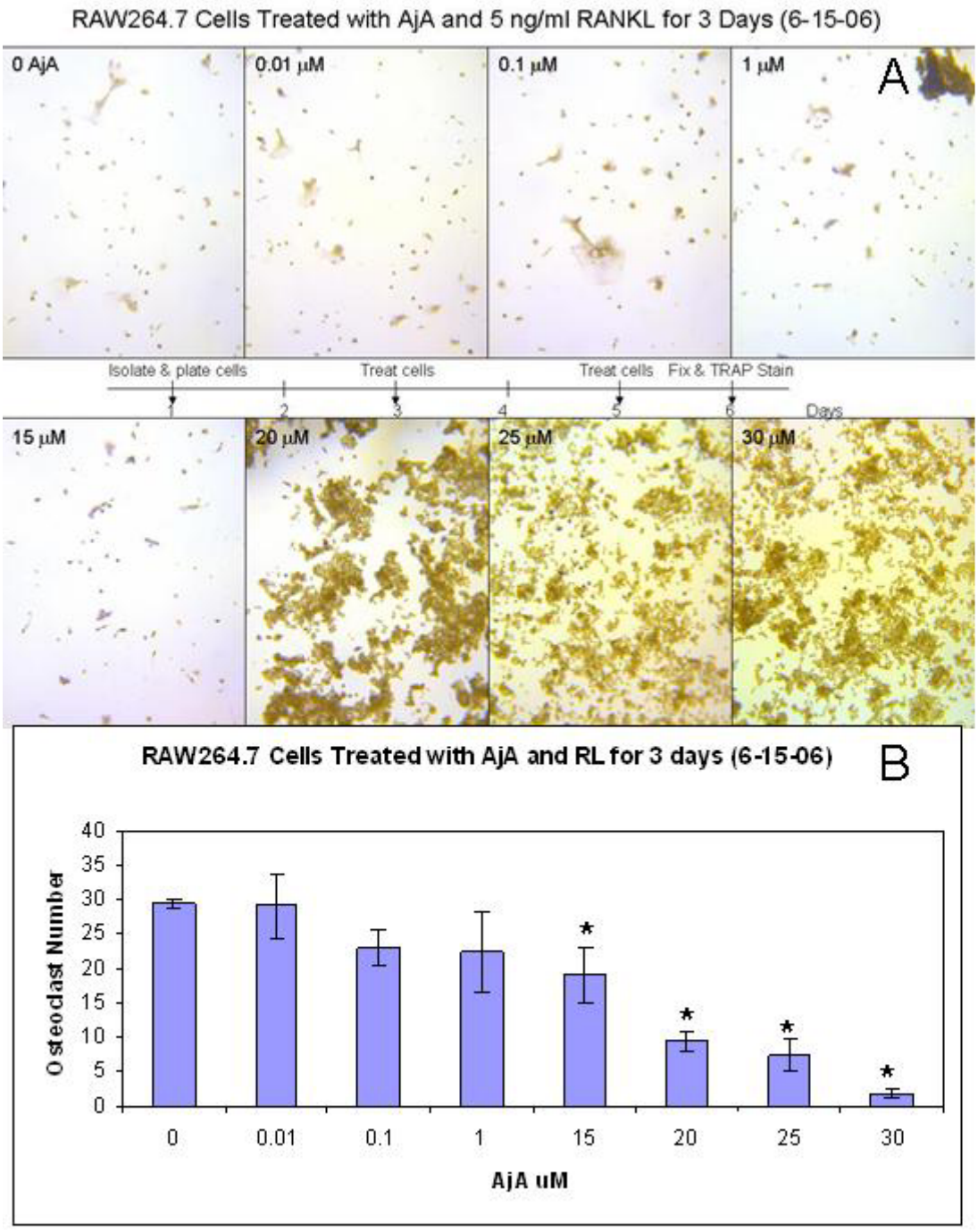
APPENDIX

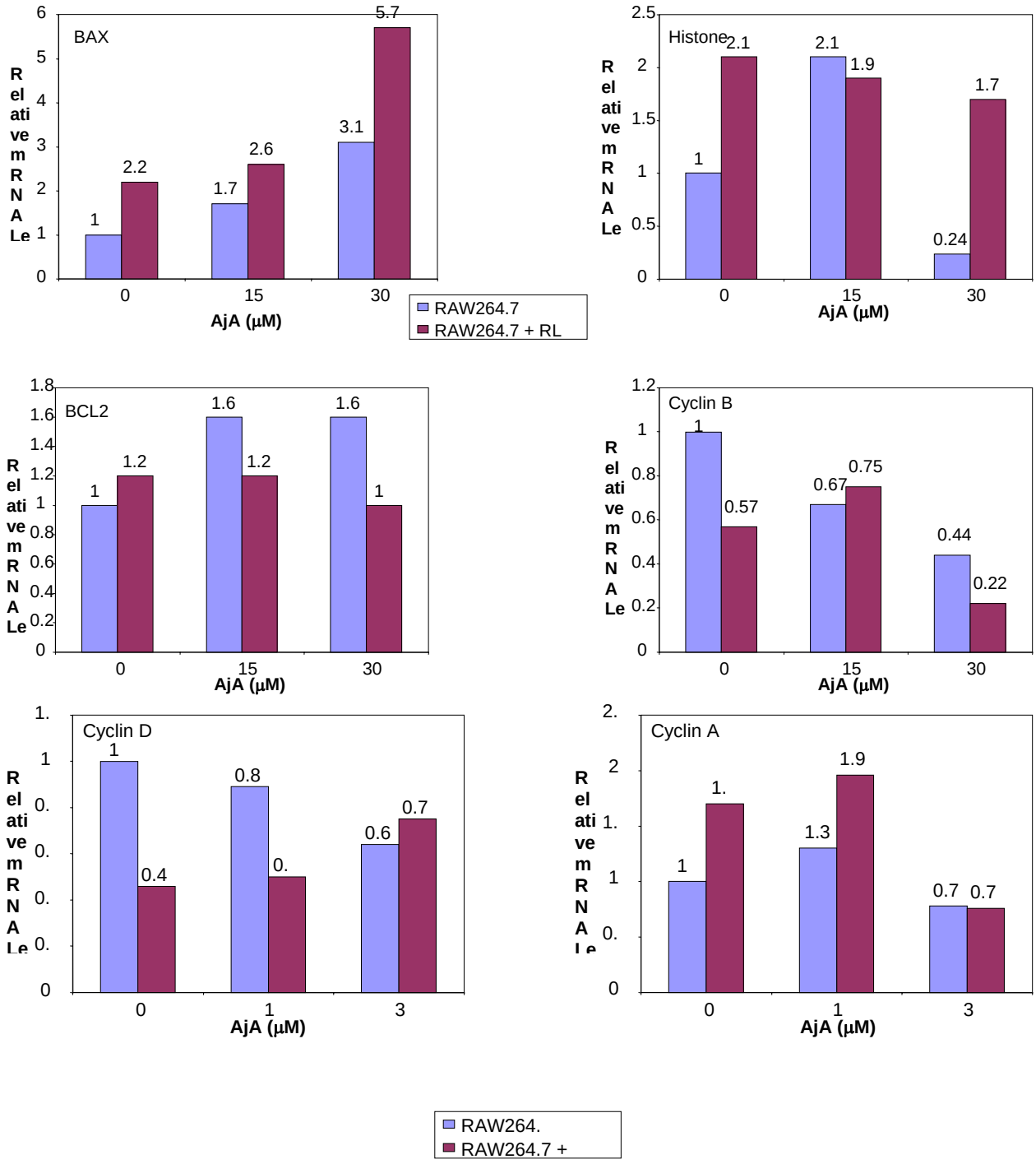


AjA suppresses osteoclastogenesis. Cytochemical detection of TRAP shown in (A) and osteoclast numbers shown in (B). The values shown are the mean ± standard deviation of triplicate fields. RANKL-treated cells were stained for TRAP and imaged.



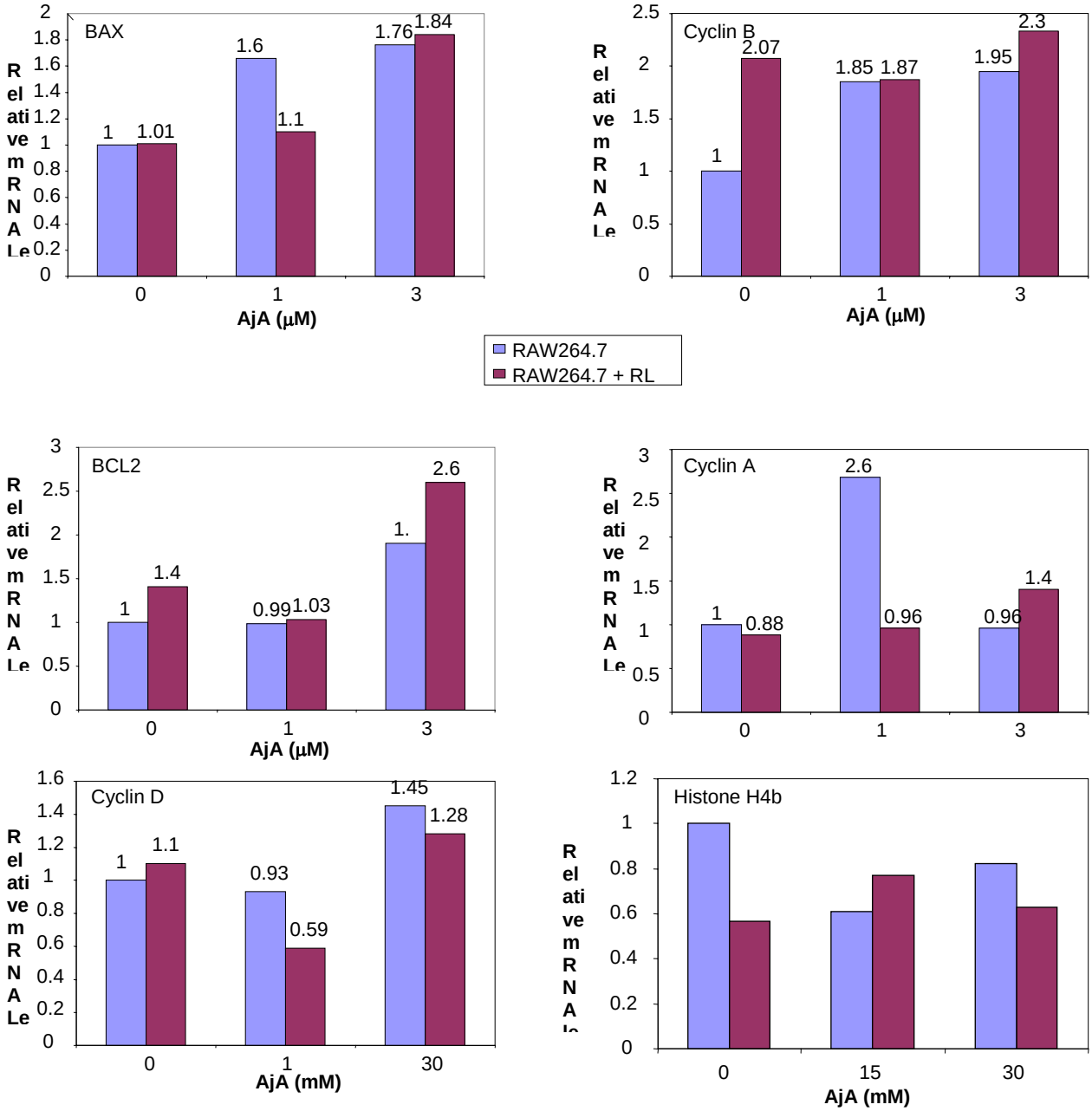
AjA suppresses osteoclastogenesis as assessed by cytochemical detection of TRAP (A) and by osteoclast numbers shown in (B). The values shown are the mean ± standard deviation of triplicate fields. RANKL-treated cells were stained for TRAP and imaged.

Effect of AjA on Gene Expression in RAW264.7 Cells (6-30-06)



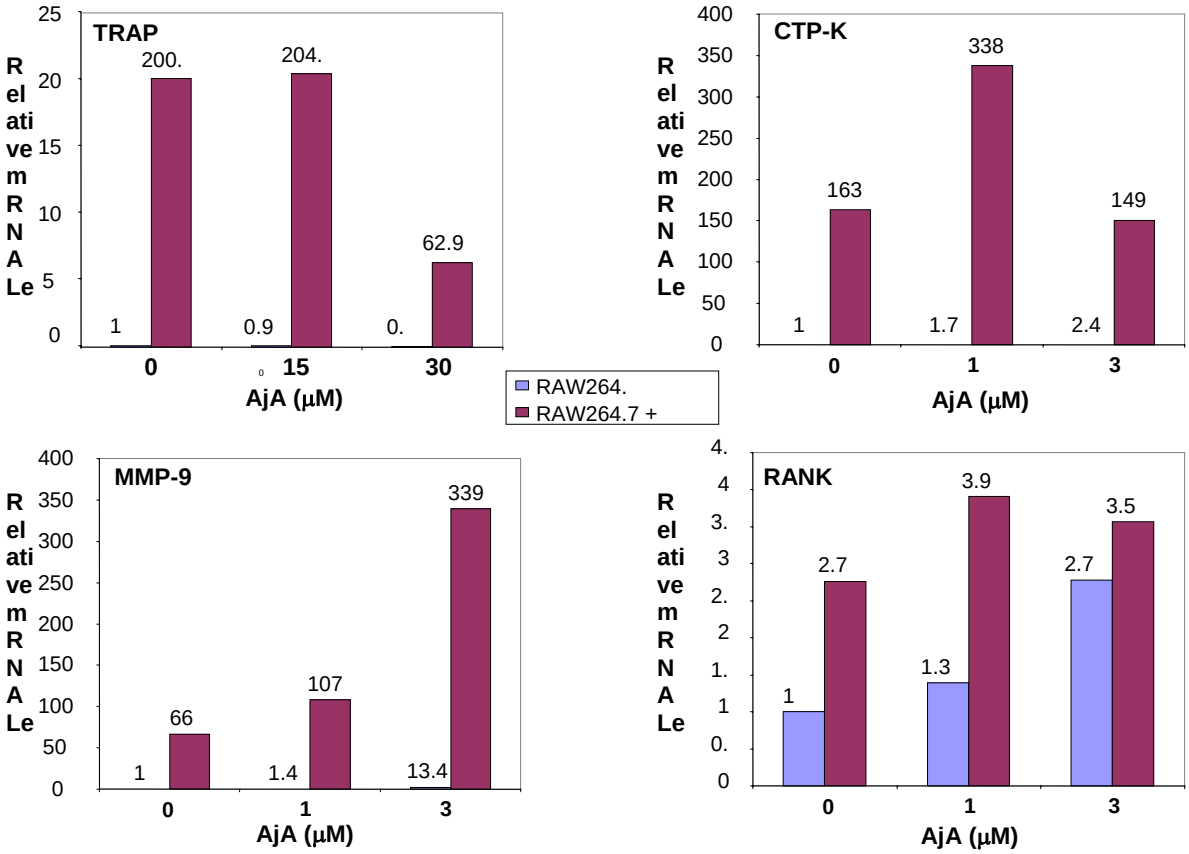
AjA suppresses osteoclastogenesis. Quantitive real time PCR of osteoclast phenotypic gene markers. Cells were cultured for 6 days in the absence (purple bar) or presence (magenta bar) of RANKL as described in "Materials and Methods." Total RNA was extracted, reverse transcribed, and subjected to real time PCR analysis using primers for known osteoclast markers. Values are normalized for GAPDH expression and expressed in arbitrary units.

Effect of AjA on Gene Expression in RAW264.7 Cells (6-21-06)



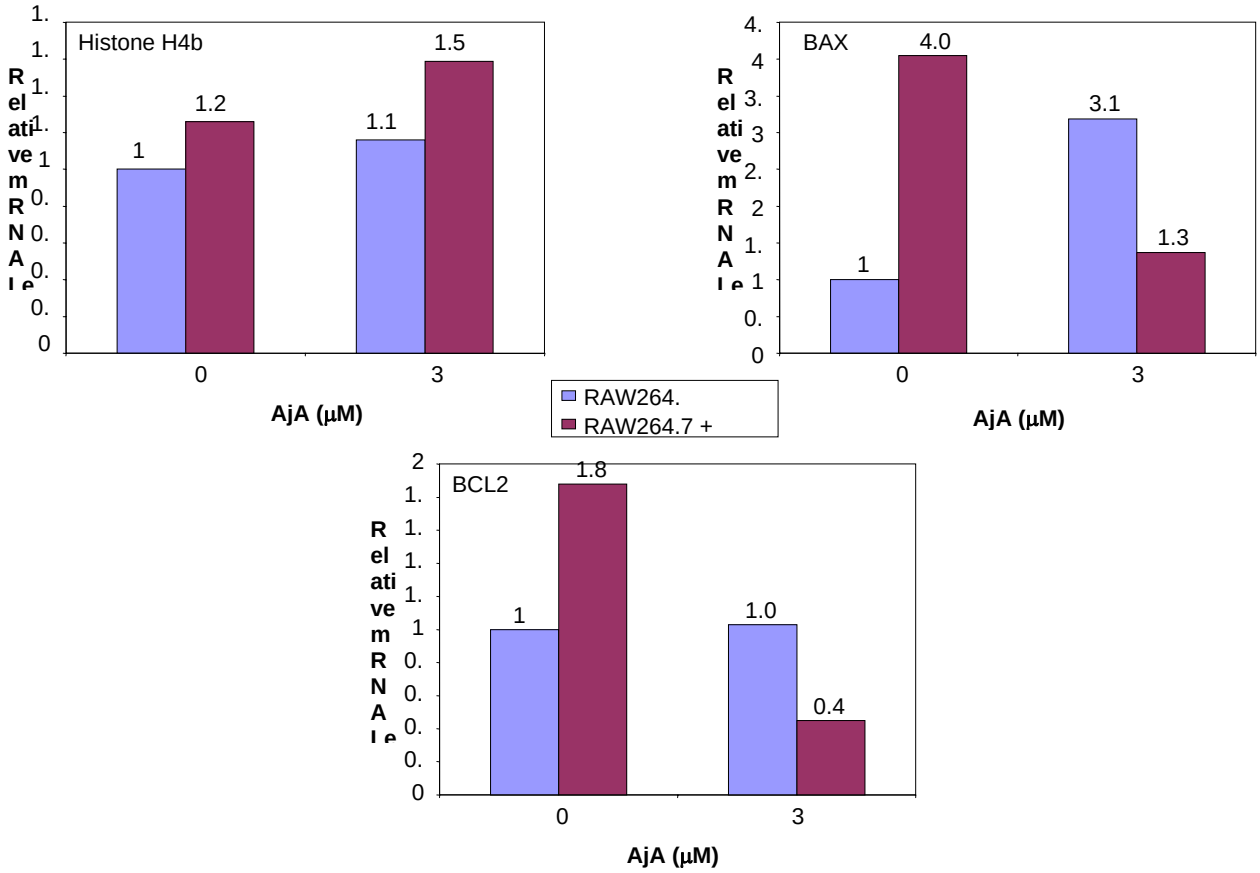
AjA suppresses osteoclastogenesis. Quantitive real time PCR of osteoclast phenotypic gene markers. Cells were cultured for 6 days in the absence (purple bar) or presence (magenta bar) of RANKL as described in "Materials and Methods." Total RNA was extracted, reverse transcribed, and subjected to real time PCR analysis using primers for known osteoclast markers. Values are normalized for GAPDH expression and expressed in arbitrary units.

Effect of AjA on Gene Expression in RAW264.7 Cells (6-19-



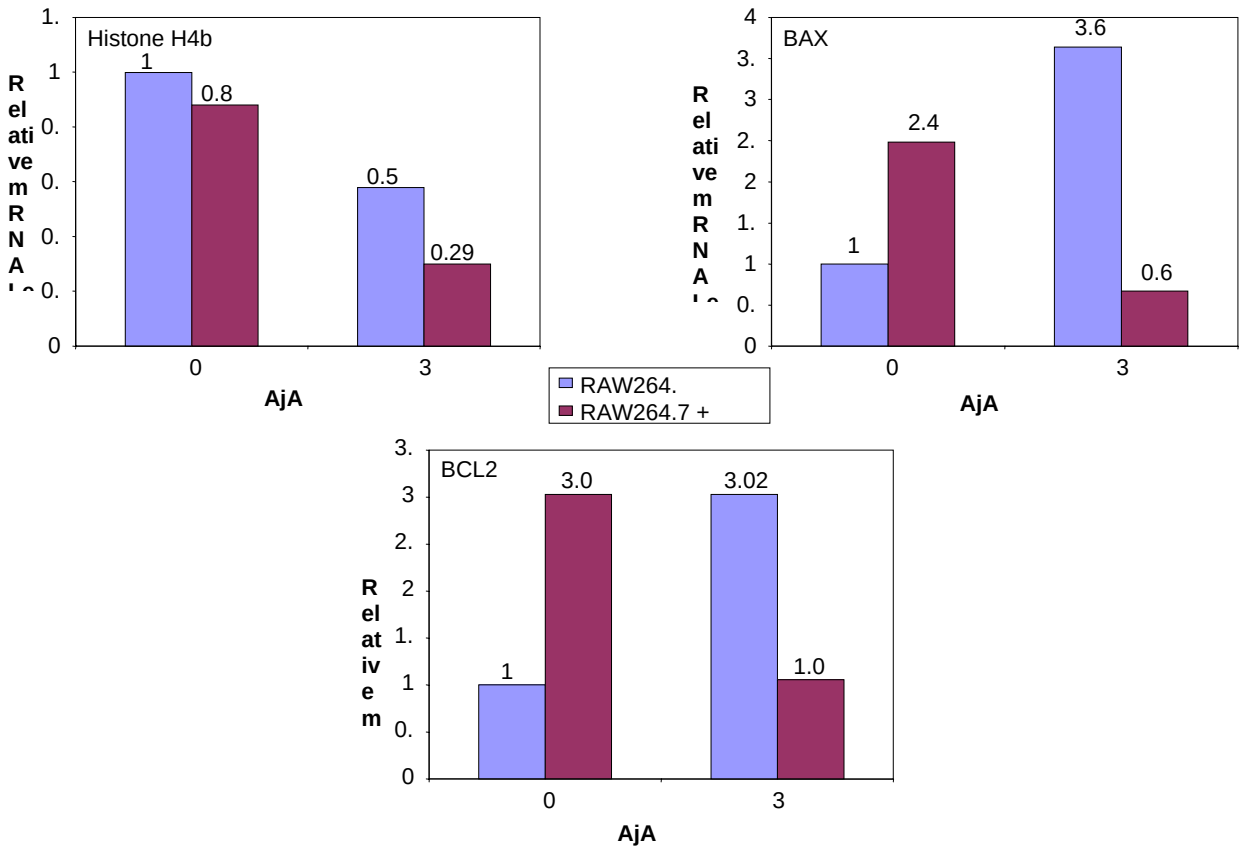
AjA suppresses osteoclastogenesis. Quantitive real time PCR of osteoclast phenotypic gene markers. Cells were cultured for 6 days in the absence (purple bar) or presence (magenta bar) of RANKL as described in "Materials and Methods." Total RNA was extracted, reverse transcribed, and subjected to real time PCR analysis using primers for known osteoclast markers. Values are normalized for GAPDH expression and expressed in arbitrary units.

Effect of AjA on Gene Expression in RAW264.7 Cells (9-12-05)



AjA suppresses osteoclastogenesis. Quantitive real time PCR of osteoclast phenotypic gene markers. Cells were cultured for 6 days in the absence (purple bar) or presence (magenta bar) of RANKL as described in "Materials and Methods." RANKL added first then AjA for the last 24 hours. Total RNA was extracted, reverse transcribed, and subjected to real time PCR analysis using primers for known osteoclast markers. Values are normalized for GAPDH expression and expressed in arbitrary units.

Effect of AjA on Gene Expression in RAW264.7 Cells (10-5-05)



AjA suppresses osteoclastogenesis. Quantitive real time PCR of osteoclast phenotypic gene markers. Cells were cultured for 6 days in the absence (purple bar) or presence (magenta bar) of RANKL as described in "Materials and Methods." RANKL added first then AjA for the last 24 hours. Total RNA was extracted, reverse transcribed, and subjected to real time PCR analysis using primers for known osteoclast markers. Values are normalized for GAPDH expression and expressed in arbitrary units.