Molecular Pharmacology Fast Forward. Published on February 24, 2006 as doi:10.1124/mol.105.020776
This article has not been copyedited and formatted. The final version may differ from this version.

MOL Manuscript # 20776

Cytosolic entry of bisphosphonate drugs requires acidification of vesicles following fluid-phase endocytosis

Keith Thompson, Michael J. Rogers, Fraser P. Coxon and Julie C. Crockett

Bone Research Group, Institute of Medical Sciences, University of Aberdeen, Aberdeen, UK.

Molecular Pharmacology Fast Forward. Published on February 24, 2006 as DOI: 10.1124/mol.105.020776
This article has not been copyedited and formatted. The final version may differ from this version.

MOL Manuscript # 20776

Running title: Bisphosphonate action requires endosomal acidification

Corresponding Author:
Dr Julie C. Crockett, Bone Research Group, Department of Medicine and Therapeutics, University of Aberdeen, Institute of Medical Sciences, Foresterhill, Aberdeen AB252ZD, UK

Telephone: +44 (0)1224 555162 Fax: +44 (0)1224 559533
E-mail: j.c.crockett@abdn.ac.uk

Number of text pages: 28
Number of tables: 0
Number of figures: 5
Number of references: 39
Number of words in:
Abstract: 239
Introduction: 510
Discussion: 1500

Non-standard abbreviations:

AF-ALN: Alendronate-AlexaFluor-488
GGPP: Geranylgeranyl diphosphate
N-BPs: Nitrogen-containing bisphosphonates
Non-N-BPs: Non-nitrogen-containing bisphosphonates

Molecular Pharmacology Fast Forward. Published on February 24, 2006 as DOI: 10.1124/mol.105.020776
This article has not been copyedited and formatted. The final version may differ from this version.

Abstract

Bisphosphonates such as alendronate and zoledronate are blockbuster drugs used to inhibit osteoclast-mediated bone resorption. Although the molecular mechanisms by which bisphosphonates affect osteoclasts are now evident, the exact route by which they are internalised by cells is not known. To clarify this, we synthesised a novel, fluorescently-labelled analogue of alendronate (AF-ALN). AF-ALN was rapidly internalised into intracellular vesicles in J774 macrophages and rabbit osteoclasts; uptake of AF-ALN or [^{14}C]zoledronate was stimulated by the presence of Ca^{2+} and Sr^{2+} and could be inhibited by addition of EGTA or clodronate, both of which chelate calcium ions. Both EGTA and clodronate also prevented the bisphosphonate-induced inhibition of Rap1A prenylation, an effect that was reversed by addition of Ca^{2+}. In J774 cells and osteoclasts, vesicular AF-ALN co-localised with dextran (but not wheatgerm agglutinin or transferrin), and uptake of AF-ALN or [^{14}C]zoledronate was inhibited by dansylcadaverine, indicating that fluid-phase endocytosis is involved in the initial internalisation of bisphosphonate into vesicles. Endosomal acidification then appears to be absolutely required for exit of bisphosphonate from vesicles and entry into the cytosol, since monensin and bafilomycin A1, both inhibitors of endosomal acidification, did not inhibit vesicular uptake of AF-ALN or internalisation of [^{14}C]zoledronate, but prevented the inhibitory effect of alendronate or zoledronate on Rap1A prenylation. Taken together, these results demonstrate that cellular uptake of bisphosphonate drugs requires fluid-phase endocytosis and is enhanced by Ca^{2+} ions, whilst transfer from endocytic vesicles into the cytosol requires endosomal acidification.

Molecular Pharmacology Fast Forward. Published on February 24, 2006 as DOI: 10.1124/mol.105.020776
This article has not been copyedited and formatted. The final version may differ from this version.

Bisphosphonates are non-hydrolysable analogues of pyrophosphate that inhibit bone resorption and have been used for more than 3 decades in the treatment of Paget's disease of bone, and more recently for the treatment of tumour-induced osteolysis, post-menopausal osteoporosis and other metabolic bone diseases (Russell and Rogers, 1999). By virtue of their ability to bind to Ca^{2+} ions, bisphosphonates rapidly localise to bone mineral *in vivo* and accumulate beneath bone-resorbing osteoclasts (Sato *et al.*, 1991;Azuma *et al.*, 1995;Masarachia *et al.*, 1996) that subsequently release and internalise the bisphosphonates in the acidic environment of the resorption lacuna. Once internalised by osteoclasts, the bisphosphonates have two distinct mechanisms of action depending on the structure of their R_2 side chain (Benford *et al.*, 1999). Nitrogen-containing bisphosphonates (N-BPs), such as alendronate and zoledronic acid (zoledronate) inhibit farnesyl diphosphate synthase, an enzyme in the mevalonate pathway (Bergstrom *et al.*, 2000;van Beek *et al.*, 1999;Dunford *et al.*, 2001). In addition to being required for cholesterol biosynthesis, the mevalonate pathway catalyses the synthesis of the isoprenoid lipids, farnesyl diphosphate and geranylgeranyldiphosphate (GGPP), which are the substrates for post-translational lipid modification (prenylation) of small GTPases such as Rap, Rac, Rho and Cdc42 (Luckman *et al.*, 1998b;Rogers, 2003). Inhibition of farnesyl diphosphate synthase prevents the synthesis of farnesyl diphosphate and GGPP, causing the accumulation of the unprenylated forms of small GTPases and thereby disrupting osteoclast function (Rogers, 2003;Coxon *et al.*, 2001). By contrast, the non-nitrogen containing bisphosphonates (non-N-BPs), such as clodronate, do not inhibit farnesyl diphosphate synthase (Dunford *et al.*, 2001) but are metabolised intracellularly by osteoclasts to non-hydrolysable analogues of ATP that induce osteoclast apoptosis (Auriola *et al.*, 1997;Frith *et al.*, 1997;Frith *et al.*, 2001).

Despite the recent major advances in understanding the molecular mechanisms of action of bisphosphonates, the mechanism by which they are internalised by osteoclasts during bone resorption is still not understood. We demonstrated recently that the non-N-BP clodronate prevented alendronate-induced apoptosis in J774 macrophage-like cells and prevented the alendronate-induced inhibition of prenylation of the small GTP-binding protein Rap1A in J774 macrophage-like cells and in rabbit osteoclasts (Frith and Rogers, 2003). We suggested that this may be the result of reduced uptake of alendronate, since clodronate also partially prevented the uptake of

Molecular Pharmacology Fast Forward. Published on February 24, 2006 as DOI: 10.1124/mol.105.020776
This article has not been copyedited and formatted. The final version may differ from this version.

[^{14}C]-labelled ibandronate. These results suggested that there may be a specific recognition step in the internalisation process.

In the present study we have investigated the mechanism by which bisphosphonates are internalised into J774 macrophage-like cells and compared this to uptake into osteoclasts. We synthesised a novel, fluorescently-labelled analogue of alendronate (AF-ALN) to visualise and quantify the uptake of alendronate into cells by confocal microscopy and flow cytometry. We then determined whether bisphosphonates or other agents that interfere with endocytosis or vesicular acidification, inhibited the cellular uptake of AF-ALN or radiolabelled zoledronate. Since flow cytometric analysis provides an indication of the total (e.g. vesicular *and* cytosolic) amount of AF-ALN within the cells we also examined the effect of these agents on bisphosphonate-induced accumulation of unprenylated Rap1A in J774 cells, an indication of the entry of N-BPs into the cytosol/peroxisomes and inhibition of farnesyl diphosphate synthase.

Materials and Methods

Reagents

Clodronate and alendronate were a kind gift of Procter and Gamble (Cincinnati, OH, USA) and the hydrated sodium salt of zoledronic acid (zoledronate) and [^{14}C]zoledronate (specific activity 1.936 GBq/mmol) were kindly provided by Novartis (Basel, Switzerland). Stock solutions of bisphosphonates were prepared in phosphate buffered saline (PBS), the pH was adjusted to 7.4 with 5M NaOH and solutions were filter-sterilised before use. Cell culture reagents were from Life Technologies (Paisley, UK). Fluorescently-labelled dextran, wheatgerm agglutinin and transferrin were from Molecular Probes (Leiden, The Netherlands). All other chemicals were purchased from Sigma (Poole, UK), unless stated otherwise.

Synthesis of fluorescently-labelled alendronate

1.13μmoles of the amine-reactive probe Alexa Fluor-488 carboxylic acid 2,3,5,6-tetrafluorophenyl ester (AF-488) (Molecular Probes, Leiden, The Netherlands) dissolved in DMSO, were mixed with 11.3μmoles alendronate (dissolved in bicarbonate buffer, pH9.0) i.e. a 1:10 molar ratio. The volume was made up to 1ml with distilled water and the solution incubated for 2 hours at room temperature with

Molecular Pharmacology Fast Forward. Published on February 24, 2006 as DOI: 10.1124/mol.105.020776
This article has not been copyedited and formatted. The final version may differ from this version.

mixing. To precipitate alendronate, 19.8μmoles $CaCl_2$ were added and the mixture centrifuged (14000*g*, 10min). The precipitate was washed 5 times in 1ml ddH_20. To bind the Ca^{2+} (and hence resolubilise the alendronate), 19.8μmoles EGTA was added to the precipitate. 100μl PBS was added until all alendronate-AlexaFluor-488 (AF-ALN) had dissolved and the solution was mixed for 30 minutes. The final solution (referred to as AF-ALN) contained approximately 7% labelled-alendronate, 93% free alendronate.

Cell culture

J774 macrophage-like cells were cultured at 37°C in Dulbecco's Modified Eagle Medium containing 10% (v/v) fetal calf serum (FCS), 100U/ml penicillin, 100μg/ml streptomycin and 2mM glutamine. For quantification of AF-ALN internalisation by flow cytometry, J774 cells were seeded into 24-well tissue culture plates at a density of 2 x 10^5 cells/well. For the measurement of [^{14}C]zoledronate internalisation, J774 cells were seeded into 12-well tissue culture plates at a density of 4 x 10^5 cells/well. For visualization of AF-ALN uptake by confocal microscopy, cells were seeded onto glass coverslips in 24 well plates at a density of 1 x 10^5 cells/well. For western blot analysis, J774 cells were seeded into 12-well plates at a density of 4 x 10^5 cells/well. In all cases, after 16 hours the culture medium was replaced with fresh medium containing test reagents and the cells were cultured for a further 4-6 hours.

Isolation and culture of rabbit osteoclasts

To visualize uptake of AF-ALN by rabbit osteoclasts, the cells were isolated as previously described (Coxon *et al.*, 2000). Briefly, the long bones from neonatal rabbits (3-4 days old) were cleaned and minced in serum-free α-modified essential medium (α-MEM). The suspension of bone chips was vortexed and, after allowing the bone chips to settle out, the bone cell suspension was plated onto glass coverslips in 24 well plates. After approximately 16 hours in culture (α-MEM containing 10% (v/v) FCS, 100U/ml penicillin, 100μg/ml streptomycin and 1mM glutamine), the cells were washed repeatedly with PBS to remove the contaminating stromal cells, then the osteoclasts were cultured in complete α-MEM.

Molecular Pharmacology Fast Forward. Published on February 24, 2006 as DOI: 10.1124/mol.105.020776
This article has not been copyedited and formatted. The final version may differ from this version.

For western blot analysis, osteoclast-like cells were generated from rabbit bone marrow using a method modified from David *et al.*, 1998. The bone cell suspension described above was seeded at a density of 5 x 10^6 cells/well in a 6-well plate in complete α-MEM supplemented with 10^{-8}M 1,25-dihydroxyvitaminD_3. The medium was replaced on day 5. When an almost confluent layer of osteoclast-like cells had formed (approximately day 10-14), the cells were washed gently with PBS to remove any remaining stromal cells. These cultures contain >95% pure multinucleated, TRAP-positive, osteoclast-like cells (Coxon *et al.*, 2003). The osteoclast-like cells were treated (in complete α-MEM) for 24 hours and then lysed for western blot analysis.

Confocal microscopy

To determine whether AF-ALN was internalised by adsorptive, receptor-mediated or fluid-phase endocytosis, cells were incubated with 1μg/ml wheatgerm agglutinin-633 (a marker for adsorptive endocytosis), 20μg/ml transferrin-633, (a marker for receptor-mediated endocytosis), or 250μg/ml tetramethylrhodamine-labelled dextran (TAMRA-dextran, a marker of fluid-phase endocytosis) together with 100μM AF-ALN. To determine whether clodronate itself had an effect on fluid-phase endocytosis, J774 cells or rabbit osteoclasts were treated with 100μM AF-ALN + 500μg/ml TAMRA-dextran (10000MW) ± 1mM clodronate. After treatment for 6 hours, the cells were washed in PBS and fixed for 10 minutes in 4% (v/v) formaldehyde. Cells were examined on a Zeiss LSM 510 Meta confocal microscope (Carl Zeiss Ltd, Welwyn Garden City, UK) and images captured using the LSM image capture software (Carl Zeiss Ltd).

Flow cytometric analysis

Flow cytometric analysis was used to quantify the uptake of AF-ALN by J774 cells over 4 hours' treatment, and to evaluate the effect of various agents on the internalization. To examine the overall accumulation of AF-ALN, J774 cells were treated with 100μM AF-ALN for 1-4 hours. For the cells treated for less than 4 hours, the AF-ALN was removed at the specified time and replaced with fresh medium. All cells were then harvested 4 hours after the start of the experiment.

Molecular Pharmacology Fast Forward. Published on February 24, 2006 as DOI: 10.1124/mol.105.020776
This article has not been copyedited and formatted. The final version may differ from this version.

To determine the effect of divalent ions (Ca^{2+}, Sr^{2+} and Mg^{2+}) on the uptake of AF-ALN, the cells were treated with 100μM AF-ALN ± 1mM $CaCl_2$, 1mM $SrCl_2$ or 1mM $MgCl_2$. To further investigate the effect of Ca^{2+} on the uptake of bisphosphonates, we measured AF-ALN uptake in the presence and absence of Ca^{2+} ions. J774 cells were treated with 100μM AF-ALN alone or together with 100-1000μM EGTA or 100-1000μM clodronate. To confirm that the effect of clodronate and EGTA on AF-ALN uptake was the result of chelation of Ca^{2+} from the medium rather than a direct cellular effect (e.g. toxicity), J774 cells were pre-treated with 1mM clodronate or 1mM EGTA for 4 hours, then cultured for a further 4 hours in fresh medium containing 100μM AF-ALN with either clodronate or EGTA, or in fresh medium containing 100μM AF-ALN alone. We also examined the effect of clodronate and EGTA on the uptake of AF-ALN in calcium-free DMEM. J774 cells were treated with 100μM AF-ALN alone or together with 1mM EGTA ± or 1mM clodronate ± 1mM $CaCl_2$ for 4 hours in calcium-free DMEM.

To examine whether endocytosis was involved in the uptake of AF-ALN, cells were treated for 4 hours with either 100μM AF-ALN or 250μg/ml FITC-dextran ± 500μM dansylcadaverine (Sigma, Poole, UK) and to examine the requirement for vesicular acidification, cells were treated for 4 hours with 100μM AF-ALN or 250μg/ml FITC-dextran ± 20μM monensin, an electroneutral monovalent ionophore (Kaiser *et al.*, 1988) or 50nM bafilomycin A1, an inhibitor of the vacuolar H^+-ATPase (Bowman *et al.*, 1988). To further clarify the endocytotic process involved in bisphosphonate internalization, J774 cells were incubated for 1 hour in the presence of 100μM AF-ALN, 250μg/ml FITC-dextran, 10μg/ml wheatgerm agglutinin-633 or 20μg/ml transferrin-633 at either 4°C or 37°C. The cells were then washed in PBS and incubated in drug-free medium at 37°C for a further 3 hours.

In all of the above experiments, cells were harvested, centrifuged (3200*g*, 5min) and washed three times in PBS. The pellet was then resuspended in 300μl 1% (v/v) formaldehyde. The samples were vortexed and analysed on a FACSCalibur (BD Biosciences) using the 488nm laser.

[^{14}C] zoledronate internalisation

J774 cells were seeded into 12-well tissue culture plates and allowed to adhere overnight. The medium was then replaced with fresh medium containing 25μM [^{14}C] zoledronate (specific activity: 1.936 GBq/mmol) ± other agents for 4h. Cells were

Molecular Pharmacology Fast Forward. Published on February 24, 2006 as DOI: 10.1124/mol.105.020776
This article has not been copyedited and formatted. The final version may differ from this version.

then washed 4 times in PBS and lysed in 50mM Tris (pH 7.7), 0.5% (w/v) deoxycholate, 1% (v/v) NP-40 and 2% (v/v) protease inhibitor cocktail (Sigma). Insoluble material was removed by centrifugation (13000g, 10 mins) and the intracellular uptake of [^{14}C]zoledronate in the soluble fraction was quantified using liquid scintillation counting. The insoluble pellet (which did not contain any radioactivity) was dissolved in 0.3M NaOH. A protein assay (Bicinchoninic Acid assay, Sigma, Poole, UK) was then carried out to allow determination of specific activity (expressed as pmoles zoledronate/mg protein).

Western blot analysis

To determine whether the entry of bisphosphonate into the cytosol of the cells was affected by any of the compounds tested by flow cytometric analysis, J774 cells were treated with 100μM alendronate or 100μM zoledronate alone or in combination with 1mM clodronate, 1mM EGTA, 1mM $CaCl_2$, 1mM $SrCl_2$ or 1mM $MgCl_2$ or with 250μM/500μM dansylcadaverine, 10μM/20μM monensin or 50nM bafilomycin A1 for 4 hours. We also examined the effects of 1mM $CaCl_2$, 1mM $SrCl_2$ or 1mM $MgCl_2$ on inhibition of Rap1A prenylation induced by treatment with 2.5μM mevastatin or a geranylgeranyl transferase inhibitor (GGTI-298, 2.5μM). The cells were lysed in 100μl radioimmunoassay precipitation (RIPA) buffer (1% Triton-X-100 [vol/vol], 0.5% sodium deoxycholate [wt/vol], 0.1% SDS [wt/vol] in PBS). Since rabbit osteoclast-like cells generated in bone marrow cultures are less sensitive to the effects of alendronate (Coxon, Crockett and Rogers, unpublished observations) than J774 cells, we examined the effect of 250μM dansylcadaverine, 10μM monensin and 25nM bafilomycin A1 on alendronate-induced inhibition of Rap1A prenylation after 24 hours' treatment (rather than after 4 hours). The osteoclast-like cells were washed in PBS, then lysed in 100μl RIPA buffer. After protein determination (Bicinchoninic Acid assay, Sigma, Poole, UK), 30μg of protein (J774 cells) or 50μg of protein (osteoclast-like cells) from each lysate were electrophoresed under reducing conditions on a 12% polyacrylamide-SDS gel (Criterion electrophoresis system, BioRad, Hemel Hempstead, UK). The proteins were transferred to polyvinyllidenedifluoride membrane, which was then incubated with 0.2μg/ml goat polyclonal anti-Rap1A antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), followed by 0.2μg/ml anti-goat IgG-HRP conjugate (Merck BioSciences,

Molecular Pharmacology Fast Forward. Published on February 24, 2006 as DOI: 10.1124/mol.105.020776
This article has not been copyedited and formatted. The final version may differ from this version.

Nottingham, UK). After visualising the chemiluminescent signal, the membrane was then incubated in rabbit polyclonal anti-actin antibody (Sigma), followed by 0.2μg/ml anti-rabbit IgG-HRP conjugate (Merck BioSciences, Nottingham, UK). The chemiluminescence was visualized using a BioRad FluorS-Max imager (BioRad, Hemel Hempstead, UK).

Statistical Analysis

Data were analysed using a oneway ANOVA with Tukey post hoc test.

Results

Ca^{2+} and Sr^{2+} ions stimulate AF-ALN uptake by J774 cells

In order to examine the pattern of uptake of AF-ALN, J774 cells that had been treated for 6 hours with 100μM AF-ALN were analysed by confocal microscopy. Green fluorescence could be observed in punctate vesicles throughout the cytosol (Fig 1A). The mean fluorescence per cell (the geomean) was quantitated by flow cytometry. When cells that had been treated for 1, 2, 3 and 4 hours were analysed, there was a time-dependent accumulation of fluorescence within the cells (Fig 1B).

To determine the effect of divalent ions on AF-ALN uptake, J774 cells were treated with 100μM AF-ALN in the presence or absence of 1mM $CaCl_2$, 1mM $SrCl_2$ or $MgCl_2$. The mean amount of fluorescence per cell increased 23-fold and 10-fold when cells were treated for 4 hours with 100μM AF-ALN + 1mM $CaCl_2$ or 1mM $SrCl_2$ respectively, compared to treatment with AF-ALN alone. By contrast, when cells were co-incubated with 1mM $MgCl_2$ there was no additional increase in AF-ALN internalisation (Fig 1C). Similar results were obtained after quantifying the uptake of [^{14}C]zoledronate in the presence of $CaCl_2$, $SrCl_2$ or $MgCl_2$ (Fig 1D). Furthermore, these observations were supported by western blot analysis of J774 cells that had been treated for 4 hours with 100μM alendronate in the presence or absence of 1mM $CaCl_2$, 1mM $SrCl_2$ or $MgCl_2$. As a result of inhibition of farnesyl diphosphate synthase, alendronate causes accumulation of unprenylated Rap1A in J774 cells, which can be detected by western blot analysis using an antibody that specifically hybridises to the unprenylated form of the small GTPase Rap1A (Frith *et al.*, 2001;Coxon *et al.*, 2001). After 4 hours' treatment with alendronate there was a slight accumulation of unprenylated Rap1A. However, this was markedly increased in

Molecular Pharmacology Fast Forward. Published on February 24, 2006 as DOI: 10.1124/mol.105.020776
This article has not been copyedited and formatted. The final version may differ from this version.

cells treated with alendronate + $CaCl_2$ or alendronate + $SrCl_2$, but not in cells treated with alendronate + $MgCl_2$ (Fig 1E). Unlike with alendronate, the addition of $CaCl_2$, $SrCl_2$ or $MgCl_2$ to the culture medium did not enhance the ability of either GGTI-298 or mevastatin to inhibit Rap1A prenylation (Fig 1E).

EGTA and clodronate inhibit the uptake and action of alendronate, which is partially reversed by Ca^{2+}

In order to investigate the requirement for Ca^{2+} ions in the uptake of AF-ALN, we examined the effect of the calcium chelator EGTA. J774 cells were treated for 4 hours in the presence of 100μM AF-ALN with or without 100μM, 250μM, 500μM or 1mM EGTA. EGTA significantly, and concentration-dependently, inhibited the uptake of AF-ALN into J774 cells, and was effective at 100μM, the lowest concentration tested, reducing the level of fluorescence per cell to 70% of the uptake of AF-ALN alone (data not shown). 1mM EGTA significantly reduced the fluorescence to 46% of the uptake of AF-ALN alone (Fig 2A).

Since we had previously described an inhibitory effect of clodronate on the uptake of radiolabelled ibandronate (Frith and Rogers, 2003), and clodronate is an effective calcium chelator (by virtue of its two phosphonate groups), we also examined whether clodronate affected AF-ALN uptake by chelating Ca^{2+}. J774 cells were treated for 4 hours in the presence of 100μM AF-ALN with or without 100μM, 250μM, 500μM or 1mM clodronate. Like EGTA, clodronate significantly, and concentration-dependently, inhibited the uptake of AF-ALN into J774 cells. 100μM clodronate reduced the level of fluorescence per cell to 59% of the uptake of AF-ALN alone (data not shown). 1mM clodronate reduced the fluorescence to 39% of the uptake of AF-ALN alone (Fig 2A). The effects of both 1mM EGTA and 1mM clodronate on AF-ALN internalisation were partially but significantly reversed by the addition of 1mM $CaCl_2$ (to 73% and 76% respectively). The same effect was also observed when we repeated these experiments using 25μM radiolabelled zoledronate instead of AF-ALN and measured the amount of radioactivity internalised in the presence or absence of 1mM EGTA or 1mM clodronate (data not shown). To further clarify whether the inhibitory effect of clodronate on AF-ALN uptake was as a result of chelation of calcium ions we repeated the above experiment using calcium-free DMEM. In the latter culture medium, the uptake of AF-ALN by J774 cells was only

Molecular Pharmacology Fast Forward. Published on February 24, 2006 as DOI: 10.1124/mol.105.020776
This article has not been copyedited and formatted. The final version may differ from this version.

55% of that observed in complete DMEM (which contains 1.8mM $CaCl_2$). Unlike in complete DMEM, 1mM EGTA and 1mM clodronate had little effect on the uptake of AF-ALN (Fig 2B). Furthermore, addition of 1mM $CaCl_2$ to calcium-free DMEM stimulated the uptake of AF-ALN to a similar level to that seen in complete DMEM and this effect of Ca^{2+} was completely reversed in the presence of clodronate or EGTA (Fig 2B).

In addition to measuring the inhibitory effect of EGTA and clodronate on AF-ALN uptake, we also examined their effect on alendronate-induced inhibition of Rap1A prenylation (a reflection of both the uptake of alendronate and the inhibition of farnesyl diphosphate synthase). 1mM EGTA or 1mM clodronate markedly reduced the inhibition of Rap1A prenylation caused by alendronate treatment (Fig 2C). This effect was largely prevented when the cells were co-incubated with 1mM $CaCl_2$. EGTA, clodronate or $CaCl_2$ alone had no effect on Rap1A prenylation (Fig 2C).

To determine whether the inhibitory effects of EGTA and clodronate on alendronate uptake/accumulation of unprenylated Rap1A were indeed as a result of Ca^{2+} chelation rather than an effect on cell metabolism or toxicity, J774 cells were pre-treated with 1mM clodronate or 1mM EGTA for 4 hours and then cultured for a further 4 hours with AF-ALN, with or without clodronate or EGTA. Both EGTA and clodronate effectively inhibited uptake of AF-ALN only when present simultaneously with AF-ALN in the culture medium (Fig 2D). When cells were only pre-treated with EGTA or clodronate, the uptake of AF-ALN was not affected.

AF-ALN colocalises with dextran, but not wheatgerm agglutinin or transferrin

To determine whether AF-ALN is internalised by adsorptive endocytosis, receptor-mediated endocytosis or fluid-phase endocytosis, we examined whether AF-ALN co-localised with fluorescently-labelled markers of each of these processes. J774 cells were incubated in the presence of 100μM AF-ALN together with 10μg/ml wheatgerm agglutinin-633, 20μg/ml transferrin-633 or 250μg/ml TAMRA-dextran for 6 hours. AF-ALN could be detected in intracellular vesicles but did not co-localise with wheatgerm agglutinin-633 (Fig 3Ai) or with transferrin-633 (Fig 3Aii). However, AF-ALN at least partially co-localised in the same vesicles as TAMRA-dextran, a marker of fluid-phase endocytosis (Fig 3Aiii).

Molecular Pharmacology Fast Forward. Published on February 24, 2006 as DOI: 10.1124/mol.105.020776
This article has not been copyedited and formatted. The final version may differ from this version.

Punctate, vesicular uptake of AF-ALN, similar to that seen in J774 cells, was also observed in mature rabbit osteoclasts (Figure 3Aiv). This pattern of uptake closely resembled that seen with TAMRA-dextran (Figure 3Av). Indeed, in rabbit osteoclasts, AF-ALN co-localised with TAMRA-dextran, but not with transferrin-633 (Fig 3Avi).

To further confirm that fluid-phase endocytosis, rather than adsorption or receptor binding, is involved in the uptake of AF-ALN, J774 cells were incubated for 1 hour with 100μM AF-ALN, 250μg/ml FITC-dextran, 10μg/ml wheatgerm agglutinin-633 or 20μg/ml transferrin-633 at 4ºC or 37ºC, then washed and incubated for 3 hours at 37ºC. The cells were then washed again, fixed and analysed by flow cytometry. The amount of fluorescence per cell was therefore a reflection of the amount of each compound bound to the cell surface during the 1 hour incubation at 4ºC or 37ºC and later internalised at 37ºC (Fig 3B). Cells that had been treated with AF-ALN or FITC-dextran for 1 hour at 4ºC accumulated 3% and 0% respectively of the amount accumulated by cells that had been incubated for 1 hour at 37ºC. By contrast, cells treated with wheatgerm agglutinin-633 or transferrin-633 at 4ºC still accumulated 30% and 35% respectively of the amount internalised at 37ºC, demonstrating that, unlike wheatgerm agglutinin or transferrin, negligible amounts of AF-ALN or dextran bind to the cell surface.

Clodronate and EGTA inhibit AF-ALN uptake without affecting fluid-phase endocytosis

The effect of clodronate on the internalisation of AF-ALN and TAMRA-dextran by J774 cells was visualised by confocal microscopy. Cells were treated with 250μg/ml TAMRA-dextran together with 100μM AF-ALN in the presence or absence of 1mM clodronate. TAMRA-dextran and AF-ALN co-localised within the same vesicles (Fig 3Ci). When the cells were co-incubated with 1mM clodronate, internalisation of AF-ALN was prevented, but the uptake of TAMRA-dextran was unaffected (Fig 3Cii). Furthermore, when the uptake of FITC-dextran over 4 hours was quantified by flow cytometry, 1mM clodronate or 1mM EGTA (which significantly inhibited the uptake of AF-ALN; Fig 2A) had no effect on the uptake of FITC-dextran, whilst 1mM $CaCl_2$ (which significantly stimulated the uptake of AF-ALN or [^{14}C]zoledronate; Fig 1C,D) had no effect on the uptake of FITC-dextran

Molecular Pharmacology Fast Forward. Published on February 24, 2006 as DOI: 10.1124/mol.105.020776
This article has not been copyedited and formatted. The final version may differ from this version.

(data not shown). Together, these observations demonstrate that chelation of Ca^{2+} ions by clodronate or EGTA inhibits the uptake of bisphosphonates by a physico-chemical mechanism rather than by reducing fluid-phase endocytosis.

Dansylcadaverine inhibits endocytic uptake of AF-ALN and [^{14}C]zoledronate

To examine the effect of dansylcadaverine, monensin and bafilomycin A1 on fluid-phase endocytosis, J774 cells were treated for 4 hours with 250μg/ml FITC-dextran in the presence or absence of 500μM dansylcadaverine, 20μM monensin or 50nM bafilomycin A1. Consistent with its known inhibitory effect on endocytosis, (Haigler *et al.*, 1980), dansylcadaverine significantly reduced the uptake of dextran by almost 75% (Fig 4A). However, neither monensin nor bafilomycin A1 had any significant effect. Similarly, 500μM dansylcadaverine significantly reduced the uptake of AF-ALN to 50% of that observed with AF-ALN alone (Fig 4B), whilst monensin or bafilomycin A1 did not inhibit the uptake of AF-ALN. The same effects were observed on the uptake of [^{14}C]zoledronate: 500μM dansylcadaverine significantly reduced the uptake of [^{14}C]zoledronate, whilst monensin or bafilomycin A1 had no effect (Fig 4C).

To confirm the lack of effect of bafilomycin A1 and monensin on vesicular uptake of AF-ALN, J774 cells that had been treated with AF-ALN with or without monensin or bafilomycin A1 were also examined by confocal microscopy. Consistent with the quantification of uptake of FITC-dextran, AF-ALN or [^{14}C]zoledronate (Fig 4A,B,C), monensin or bafilomycin A1 did not affect endocytosis of AF-ALN into punctate vesicles (Fig 4D).

Dansylcadaverine, monensin and bafilomycin A1 prevent bisphosphonate-induced inhibition of Rap1A prenylation

By contrast with the measurement of AF-ALN uptake by flow cytometry, which gives an indication of the total amount of intracellular AF-ALN (e.g. cytosolic *and* vesicular), analysis of Rap1A prenylation demonstrates effects on exit of bisphosphonates from the endosomes into other intracellular compartments where farnesyl diphosphate synthase resides. Farnesyl diphosphate synthase is synthesised within the cytosol and is translocated post-translationally into peroxisomes (Olivier *et al.*, 2000). In agreement with the flow cytometric analyses, dansylcadaverine partially

Molecular Pharmacology Fast Forward. Published on February 24, 2006 as DOI: 10.1124/mol.105.020776
This article has not been copyedited and formatted. The final version may differ from this version.

prevented the alendronate-induced accumulation of unprenylated Rap1A in a concentration-dependent manner (Fig 5A). The same effect was observed when J774 cells were treated with 100μM zoledronate + dansylcadaverine. Like alendronate, 100μM zoledronate caused an accumulation of unprenylated Rap1A, which was partially, and concentration-dependently, prevented when the cells were co-incubated with 250μM or 500μM dansylcadaverine (Fig 5A).

By contrast to the lack of effects seen on the uptake of AF-ALN, either monensin or bafilomycin A1 completely prevented the accumulation of unprenylated Rap1A when J774 cells were treated with 100μM alendronate or zoledronate (Fig 5A). J774 cells accumulated the acidotropic probe LysoTracker Red (Molecular Probes) within intracellular vesicles in untreated J774 cells, but not in cells treated with 50nM bafilomycin A1 or 20μM monensin (data not shown), indicating that both bafilomycin A1 and monensin, at concentrations that blocked the inhibitory effect of N-BPs on protein prenylation, prevented the acidification of intracellular organelles.

In agreement with the data obtained in J774 cells, 10μM monensin, 25nM bafilomycin A1 or 250μM dansylcadaverine prevented the inhibitory effect of alendronate on Rap1A prenylation in rabbit osteoclast-like cells (Figure 5B).

Discussion

Bisphosphonate drugs are effective calcium chelators and, after oral or *iv* administration, rapidly target the skeleton where they inhibit osteoclast-mediated bone resorption (Rogers, 2003). During bone resorption, acid (H^+ and Cl^- ions) and proteolytic enzymes are actively secreted across the osteoclast ruffled border membrane (Sundquist *et al.*, 1990). It is thought that, in the acidic pH of the resorption lacuna, protonation of the phosphonate groups of bisphosphonates causes their release from bone surfaces into solution (Ebetino *et al.*, 1998). However, the exact route by which bisphosphonate drugs are then internalised by osteoclasts (and possibly other cells in the bone microenvironment) remains unknown. In this study, we examined the mechanism of internalisation of bisphosphonates by studying the uptake of radiolabelled zoledronate and a novel, fluorescently-labelled analogue of alendronate (AF-ALN) into J774 macrophages and rabbit osteoclasts. J774 cells were used because the structure-activity relationships of bisphosphonates for reducing J774 cell viability closely matches the structure-activity relationships for inhibiting bone

Molecular Pharmacology Fast Forward. Published on February 24, 2006 as DOI: 10.1124/mol.105.020776
This article has not been copyedited and formatted. The final version may differ from this version.

resorption *in vivo* (Luckman *et al.*, 1998a), due to effects on the same intracellular molecular target, farnesyl diphosphate synthase (Dunford *et al.*, 2001).

In macrophages and osteoclasts, AF-ALN appeared to be internalised from solution initially by fluid-phase endocytosis, since the punctate, vesicular fluorescence staining (Fig 1) colocalised with a marker of fluid-phase endocytosis (FITC-dextran), but not with markers of adsorptive or receptor-mediated endocytosis (wheatgerm agglutinin or transferrin, respectively) (Fig 3). Like with FITC-dextran, we could not detect appreciable binding of AF-ALN to cells incubated at 4^{o}C (unlike wheatgerm agglutinin or transferrin), suggesting the lack of any receptor-binding or adsorptive step prior to endocytosis of AF-ALN (Fig 3). The rapid internalisation of AF-ALN, and continued accumulation over several hours (Fig 1), is also consistent with the rapid rate of fluid-phase endocytosis of these cells. Furthermore, the vesicular uptake of AF-ALN was at least partially prevented by an inhibitor of fluid-phase endocytosis, dansylcadaverine (Schlegel *et al.*, 1982;Davies *et al.*, 1980), which also prevented uptake of FITC-dextran (Fig 4). This route of uptake of AF-ALN was not an artefact due to modification of a bisphosphonate by addition of a bulky fluorophore, since dansylcadaverine also prevented the internalisation of [^{14}C]zoledronate, and concentration-dependently prevented the inhibitory effect of two unlabelled N-BPs (alendronate and zoledronate) on the prenylation of Rap1A. The latter effect involves inhibition of the target enzyme farnesyl diphosphate synthase, which is synthesised in the cytosol and translocated, probably as the folded protein, into peroxisomes (McNew and Goodman, 1994;Olivier *et al.*, 2000). Together, these observations demonstrate that initial internalisation by fluid-phase endocytosis is a prerequisite for the pharmacological action of N-BPs.

To determine how N-BPs translocate from endocytic vesicles into the cytoplasm, we examined the effect of inhibitors of endosomal acidification. Monensin is a cationic ionophore that prevents acidification of endosomes (Kaiser *et al.*, 1988) and bafilomycin A1 is an inhibitor of the vacuolar H^{+}-ATPase (Bowman *et al.*, 1988). Neither monensin nor bafilomycin A1 had any detectable inhibitory effect on the total uptake of AF-ALN, FITC-dextran or [^{14}C]zoledronate into J774 cells (Fig 4). However, monensin and bafilomycin A1 both prevented the inhibitory effect of alendronate or zoledronate on Rap1A prenylation in macrophages and osteoclasts. This demonstrates that, although monensin and bafilomycin A1 do not inhibit the internalisation of bisphosphonate into intracellular vesicles, they prevent entry of the

Molecular Pharmacology Fast Forward. Published on February 24, 2006 as DOI: 10.1124/mol.105.020776
This article has not been copyedited and formatted. The final version may differ from this version.

drugs into the cytosol by preventing acidification of endocytic vesicles. At low pH, protonation of the phosphonate groups of bisphosphonates would reduce the anionic charge of the compounds, thereby releasing any bound Ca^{2+} ions and probably allowing diffusion of the bisphosphonates across the vesicular membrane into the cytoplasm (although we cannot rule out the possibility that bisphosphonates traverse the vesicular membrane via a transport protein). Our observation that bafilomycin A1 and monensin prevent the inhibitory effect of alendronate or zoledronate on protein prenylation in macrophages and osteoclasts *in vitro* are consistent with a recent study demonstrating that bafilomycin A1 prevented the disruptive effect of the bisphosphonates risedronate and etidronate on cytoskeletal organisation of osteoclasts *in vitro* (Takami *et al.*, 2003). However, in the latter study, the authors incorrectly concluded that extracellular acidification was necessary for internalization of bisphosphonates across the plasma membrane of osteoclasts in the absence of bone mineral. We clearly demonstrate that, in fact, internalization by fluid-phase endocytosis is first required (which is inhibited by dansylcadaverine), followed by acidification of endocytic vesicles.

The lack of an effect of bafilomycin A1 or monensin on the total amount of intracellular AF-ALN or [^{14}C]zoledronate (Fig 4), despite complete inhibition of the effects of alendronate or zoledronate on protein prenylation (Fig 5), indicates that the amount of N-BP in the cytosol is negligible compared to the amount present in endocytic vesicles. Since N-BPs such as zoledronate inhibit farnesyl diphosphate synthase at nanomolar and even picomolar concentrations (Dunford *et al.*, 2001), very low concentrations achieved in the cytoplasm might still be sufficient to inhibit farnesyl diphosphate synthase in peroxisomes. This enzyme is synthesised in the cytosol on free ribosomes and then imported into peroxisomes. Most proteins destined for the peroxisomal matrix are translocated in their mature conformations (McNew and Goodman, 1996). It is therefore possible that bisphosphonates associate with farnesyl diphosphate synthase in the cytosol and are then imported into the peroxisomes together with newly-synthesised farnesyl diphosphate synthase. Some proteins that lack a specific peroxisome targeting sequence have also been demonstrated to “piggy-back” onto other proteins to gain access to the peroxisomes (McNew and Goodman, 1994).

Finally, since a previous study by Mönkkönen et al (Mönkkönen *et al.*, 1994) demonstrated that Ca^{2+} ions enhanced the inhibitory effect of bisphosphonates on

Molecular Pharmacology Fast Forward. Published on February 24, 2006 as DOI: 10.1124/mol.105.020776
This article has not been copyedited and formatted. The final version may differ from this version.

RAW264 cell proliferation, we examined the role of Ca^{2+} ions on the uptake of N-BPs. Consistent with these previous observations, we found that the presence of 1mM Ca^{2+} or Sr^{2+} (but not Mg^{2+}) increased the uptake of AF-ALN or [^{14}C]zoledronate and enhanced the inhibitory effect of alendronate on protein prenylation (Fig 1). Furthermore, addition of EGTA or a molar excess of the bisphosphonate clodronate, which does not inhibit protein prenylation (Luckman *et al.*, 1998b;Dunford *et al.*, 2001), significantly reduced (but did not entirely inhibit) the uptake of AF-ALN and reduced the inhibitory effect of alendronate on protein prenylation (Fig 2). The latter effects of EGTA or clodronate were due to chelation of Ca^{2+}, since clodronate or EGTA had to be present simultaneously with the AF-ALN and the effects could be overcome by the further addition of Ca^{2+}(Fig 2). In addition, when J774 cells were cultured in calcium-free medium, AF-ALN uptake decreased to approximately half of that seen in complete DMEM and was not decreased further by the addition of clodronate or EGTA. Furthermore, clodronate did not affect the uptake of FITC-dextran (Fig 3). Together, these observations demonstrate that the presence of Ca^{2+} or Sr^{2+} ions enhances the endocytic internalisation of N-BPs by some physicochemical mechanism. Indeed, it has been reported that Ca^{2+} promotes the aggregation of precipitable, polymeric complexes with bisphosphonates (Matczak-Jon *et al.*, 2002). This also explains our earlier finding that clodronate could prevent the inhibitory effect of the N-BP ibandronate on protein prenylation (Frith and Rogers, 2003) (by chelating Ca^{2+} and reducing ibandronate uptake). Unlike Ca^{2+} and Sr^{2+} ions, Mg^{2+} did not appear to stimulate uptake of AF-ALN (Fig 1) or enhance the effect of alendronate on Rap1A prenylation. Although bisphosphonates can chelate Mg^{2+} ions, the latter are less likely to form multinuclear complexes with bisphosphonates since Mg^{2+} ions are smaller and less flexible than Ca^{2+} (Matczak-Jon and Videnova-Adrabinska, 2005).

In conclusion, we provide the first conclusive evidence that cellular internalisation of bisphosphonate drugs is dependent on fluid-phase endocytosis and vesicular acidification. *In vivo*, bisphosphonates are rapidly cleared from the circulation (Lin, 1996) and bind to Ca^{2+}-containing bone mineral surfaces at sites of active bone remodelling, particularly areas undergoing osteoclastic resorption (Masarachia *et al.*, 1996). Since the ability to chelate Ca^{2+} is reduced at acidic pH (Ebetino *et al.*, 1998), bisphosphonate bound to bone mineral is released from the bone surface in the acidic environment of the resorption lacuna beneath the resorbing

osteoclast, giving rise to a concentrated, localised drug solution (Rogers, 2003). Our findings suggest that bisphosphonate is probably then internalised into osteoclasts (perhaps as complexes with Ca^{2+}) by fluid-phase endocytosis of the extracellular fluid. Consistent with this, radiolabelled bisphosphonate has been detected by microautoradiography within endocytic vacuoles in resorbing osteoclasts (Sato *et al.*, 1991;Masarachia *et al.*, 1996). Vacuolar-type H^+-ATPase is also highly abundant in osteoclasts (Vaananen *et al.*, 1990), thereby allowing acidification of endocytic vesicles and entry of bisphosphonate into the osteoclast cytosol.

In addition to their known ability to affect osteoclasts, there is considerable interest in the potential anti-tumour activity of N-BPs *in vivo*, via direct effects on tumour cells or on other tumour-associated cells such as endothelial cells or infiltrating macrophages (Green, 2004;Giraudo *et al.*, 2004). Although bisphosphonates can affect a wide variety of cell types *in vitro* (Rogers, 2003;Green, 2004), our studies suggest that the ability of bisphosphonates to affect cells other than osteoclasts *in vivo* may be determined by their endocytic capacity, as well as by the concentration of available bisphosphonate in the extracellular fluid.

Acknowledgments

We thank Dr. Jonathan Green (Novartis, Pharma AG, Basel, Switzerland) for useful discussions and Miss Danielle Scheven for her contribution to this work.

References

Auriola S, Frith J, Rogers MJ, Koivuniemi A and Mönkkönen J (1997) Identification of adenine nucleotide-containing metabolites of bisphosphonate drugs using ion-pair liquid chromatography- electrospray mass spectrometry. *J Chrom B* **704**: 187-195.

Azuma Y, Sato H, Oue Y, Okabe K, Ohta T, Tsuchimoto M and Kiyoki M (1995) Alendronate distributed on bone surfaces inhibits osteoclastic bone resorption in vitro and in experimental hypercalcemia models. *Bone* **16**: 235-245.

Benford HL, Frith JC, Auriola S, Mönkkönen J and Rogers MJ (1999) Farnesol and geranylgeraniol prevent activation of caspases by aminobisphosphonates: biochemical evidence for two distinct pharmacological classes of bisphosphonate drugs. *Mol Pharmacol* **56**: 131-140.

Bergstrom JD, Bostedor RG, Masarachia PJ, Reszka AA and Rodan G (2000) Alendronate is a specific, nanomolar inhibitor of farnesyl diphosphate synthase. *Arch Biochem Biophys* **373**: 231-241.

Bowman EJ, Siebers A and Altendorf K (1988) Bafilomycins: a class of inhibitors of membrane ATPases from microorganisms, animal cells, and plant cells. *Proc Natl Acad Sci U S A* **85**: 7972-7976.

Coxon FP, Frith JC, Benford HL and Rogers MJ (2003) Isolation and purification of rabbit osteoclasts. *Methods Mol Med* **80**: 89-99.

Coxon FP, Helfrich MH, Larijani B, Muzylak M, Dunford JE, Marshall D, McKinnon AD, Nesbitt SA, Horton MA., Seabra MC., Ebetino FH., and Rogers MJ (2001) Identification of a novel phosphonocarboxylate inhibitor of Rab geranylgeranyl

transferase that specifically prevents Rab prenylation in osteoclasts and macrophages. *J Biol Chem* 276: 48213-48222.

Coxon FP, Helfrich MH, van 't Hof RJ, Sebti SM, Ralston SH, Hamilton AD and Rogers MJ (2000) Protein geranylgeranylation is required for osteoclast formation, function, and survival: inhibition by bisphosphonates and GGTI-298. *J Bone Miner Res* **15**: 1467-1476.

David JP, Neff L, Chen Y, Rincon M, Horne WC and Baron R (1998) A new method to isolate large numbers of rabbit osteoclasts and osteoclast-like cells: application to the characterization of serum response element binding proteins during osteoclast differentiation. *J Bone Miner Res* **13**: 1730-1738.

Davies PJ, Davies DR, Levitzki A, Maxfield FR, Milhaud P, Willingham MC and Pastan IH (1980) Transglutaminase is essential in receptor-mediated endocytosis of alpha 2-macroglobulin and polypeptide hormones. *Nature* **283**: 162-167.

Dunford JE, Thompson K, Coxon FP, Luckman SP, Hahn FM, Poulter CD, Ebetino FH and Rogers MJ (2001) Structure-activity relationships for inhibition of farnesyl diphosphate synthase *in vitro* and inhibition of bone resorption *in vivo* by nitrogen-containing bisphosphonates. *J Pharmacol Exp Ther* **296**: 235-242.

Ebetino FH, Francis MD, Rogers MJ and Russell RGG (1998) Mechanisms of action of etidronate and other bisphosphonates. *Rev Contemp Pharmacother* **9**: 233-243.

Frith JC, Mönkkönen J, Auriola S, Mönkkönen H and Rogers MJ (2001) The molecular mechanism of action of the anti-resorptive and anti-inflammatory drug clodronate: evidence for the formation in vivo of a metabolite that inhibits bone

resorption and causes osteoclast and macrophage apoptosis. *Arthritis Rheum* **44**: 2201-2210.

Frith JC, Mönkkönen J, Blackburn GM, Russell RG and Rogers MJ (1997) Clodronate and liposome-encapsulated clodronate are metabolized to a toxic ATP analog, adenosine 5'-(beta, gamma- dichloromethylene) triphosphate, by mammalian cells in vitro. *J Bone Miner Res* **12**: 1358-1367.

Frith JC and Rogers M J (2003) Antagonistic effects of different classes of bisphosphonates in osteoclasts and macrophages in vitro. *J Bone Miner Res* **18**: 204-212.

Giraudo E, Inoue M and Hanahan D (2004) An amino-bisphosphonate targets MMP-9-expressing macrophages and angiogenesis to impair cervical carcinogenesis. *J Clin Invest* **114**: 623-633.

Green JR (2004) Bisphosphonates: preclinical review. *Oncologist* **9 Suppl 4**: 3-13.

Haigler HT, Maxfield FR, Willingham MC and Pastan I (1980) Dansylcadaverine inhibits internalization of 125I-epidermal growth factor in BALB 3T3 cells. *J Biol Chem* **255**: 1239-1241.

Kaiser J, Stockert R J and Wolkoff A W (1988) Effect of monensin on receptor recycling during continuous endocytosis of asialoorosomucoid. *Exp Cell Res* **174**: 472-480.

Lin JH (1996) Bisphosphonates: a review of their pharmacokinetic properties. *Bone* **18**: 75-85.

Luckman SP, Coxon FP, Ebetino FH, Russell RGG and Rogers MJ (1998a) Heterocycle-containing bisphosphonates cause apoptosis and inhibit bone resorption by preventing protein prenylation: evidence from structure- activity relationships in J774 macrophages. *J Bone Miner Res* **13**: 1668-1678.

Luckman SP, Hughes DE, Coxon FP, Russell RGG and Rogers MJ (1998b) Nitrogen-containing bisphosphonates inhibit the mevalonate pathway and prevent post-translational prenylation of GTP-binding proteins, including Ras. *J Bone Miner Res* **13**: 581-589.

Masarachia P, Weinreb M, Balena R and Rodan G A (1996) Comparison of the distribution of 3H-alendronate and 3H- etidronate in rat and mouse bones. *Bone* **19**: 281-290.

Matczak-Jon E, Kurzak B, Kamecka A and Karfarski P. (2002) Interations of zinc(II), magnesium(II) and calcium(II) with aminomethane-1,1-disphosphonic acids in aqueous solutions. *Polyhedron* **21**:321-332.

Matczak-Jon E and Videnova-Adrabinska V (2005) Supramolecular chemistry and complexation abilities of diphosphonic acids. *Coordination Chemistry Reviews* **249**: 2458-2488.McNew JA and Goodman JM (1994) An oligomeric protein is imported into peroxisomes in vivo. *J Cell Biol* **127**: 1245-1257.

McNew JA and Goodman JM (1996) The targeting and assembly of peroxisomal proteins: some old rules do not apply. *Trends Biochem Sci* **21**: 54-58.

Mönkkönen J, Taskinen M, Auriola SO and Urtti A (1994) Growth inhibition of macrophage-like and other cell types by liposome-encapsulated, calcium-bound, and free bisphosphonates in vitro. *J Drug Targ* **2**: 299-308.

Olivier LM, Kovacs W, Masuda K, Keller GA and Krisans SK (2000) Identification of peroxisomal targeting signals in cholesterol biosynthetic enzymes. AA-CoA Thiolase, HMG-CoA Synthase, MPPD, and FPP synthase. *J Lipid Res* **41**: 1921-1935.

Rogers MJ (2003) New insights into the molecular mechanisms of action of bisphosphonates. *Curr Pharm Des* **9**: 2643-2658.

Russell RGG and Rogers MJ (1999) Bisphosphonates: from the laboratory to the clinic and back again. *Bone* **25**: 97-106.

Sato M, Grasser W, Endo N, Akins R, Simmons H, Thompson DD, Golub E and Rodan G A (1991) Bisphosphonate action. alendronate localization in rat bone and effects on osteoclast ultrastructure. *J Clin Invest* **88**: 2095-2105.

Schlegel R, Dickson RB, Willingham MC and Pastan IH (1982) Amantadine and dansylcadaverine inhibit vesicular stomatitis virus uptake and receptor-mediated endocytosis of alpha 2-macroglobulin. *Proc Natl Acad Sci U S A* **79**: 2291-2295.

Sundquist K, Lakkakorpi P, Wallmark B and Vaananen K (1990) Inhibition of osteoclast proton transport by bafilomycin A1 abolishes bone resorption. *Biochem Biophys Res Commun* **168**: 309-313.

Takami M, Suda K, Sahara T, Itoh K, Nagai K, Sasaki T, Udagawa N and Takahashi N (2003) Involvement of vacuolar H+ -ATPase in incorporation of risedronate into osteoclasts. *Bone* **32**: 341-349.

Vaananen HK, Karhukorpi EK, Sundquist K, Wallmark B, Roininen I, Hentunen T, Tuukkanen J and Lakkakorpi P (1990) Evidence for the presence of a proton pump of

the vacuolar H(+)-ATPase type in the ruffled borders of osteoclasts. *J Cell Biol* **111**: 1305-1311.

van Beek E, Pieterman E, Cohen L, Lowik C and Papapoulos S (1999) Farnesyl pyrophosphate synthase is the molecular target of nitrogen-containing bisphosphonates. *Biochem Biophys Res Commun* **264**: 108-111.

Footnotes

This work was funded by a grant from Novartis, Pharma AG, Basel, Switzerland.

This work has been presented in part: Thompson *et al*, (2005) Bone, 36 S302-S303 Suppl. 2.

Address for reprint requests:

Dr Julie C. Crockett, Bone Research Group, Department of Medicine and Therapeutics, University of Aberdeen, Institute of Medical Sciences, Foresterhill, Aberdeen AB252ZD, UK. E-mail: j.c.crockett@abdn.ac.uk

Molecular Pharmacology Fast Forward. Published on February 24, 2006 as DOI: 10.1124/mol.105.020776
This article has not been copyedited and formatted. The final version may differ from this version.

Figure Legends

Figure 1: Internalisation of AF-ALN by J774 cells. A) J774 cells were incubated for 6 hours in the presence of 100μM AF-ALN, then fixed and analysed by confocal microscopy (bar = 10μm). B) Cells were incubated for 1-4 hours with 100μM AF-ALN, or C) for 4 hours with 100μM AF-ALN alone or with 1mM $CaCl_2$, 1mM $SrCl_2$ or 1mM $MgCl_2$, then fixed and analysed by flow cytometry. Results shown are mean (± SEM) of 4 independent experiments. D) J774 cells were incubated for 4 hours with 25μM [^{14}C]zoledronate (ZOL) alone or with 1mM $CaCl_2$, 1mM $SrCl_2$ or 1mM $MgCl_2$, lysed then internalised [^{14}C]zoledronate quantified by liquid scintillation counting. Results shown are mean (± SEM) of 4 independent experiments. ** = $p<0.01$ compared to 25μM [^{14}C]zoledronate alone. E) Cells were incubated for 4 hours with 100μM alendronate (ALN), 2.5μM mevastatin (MEV) or 2.5μM GGTI-298 ± 1mM $CaCl_2$, 1mM $SrCl_2$ or 1mM $MgCl_2$ then analysed by western blotting for unprenylated Rap1A and β-actin. The data shown are representative of 3 independent experiments.

Figure 2: Internalisation of AF-ALN and alendronate into J774 cells is reduced by removal of Ca^{2+}. J774 cells were incubated for 4 hours with 100μM AF-ALN alone or in the presence of 1mM EGTA or clodronate, ± 1mM $CaCl_2$, in A) complete DMEM (containing 1.8mM $CaCl_2$) or B) calcium-free DMEM, then fixed and analysed by flow cytometry. Results are mean (± SEM) of 4 independent experiments. * = $p<0.05$ compared to 100μM AF-ALN alone, † = $p<0.05$ compared to treatments without $CaCl_2$. C) J774 cells were incubated with 100μM alendronate (ALN), 1mM clodronate (CLO) or 1mM EGTA alone or with 100μM alendronate ± 1mM EGTA or 1mM clodronate, in the presence or absence of 1mM $CaCl_2$, then analysed by western blotting for unprenylated Rap1A and β-actin. The data shown are representative of 3

independent experiments. In the experiment shown, the ratio of unprenylated Rap1A:β-actin was 2.79 with 100μM alendronate, and was reduced to 1.00 and 0.71 by clodronate and EGTA respectively. Ca^{2+} reversed this effect on Rap1A prenylation to ratios of 1.66 (EGTA) and 1.80 (clodronate). D) J774 cells were pre-treated for 4 hours with 1mM EGTA, 1mM clodronate or PBS. The cells were then washed in fresh medium and incubated for 4 hours with either 100μM AF-ALN alone or with 1mM EGTA or 1mM clodronate, then fixed and analysed by flow cytometry. Results are mean (± SEM) of 3 independent experiments, * = $p<0.05$ compared to treatment with AF-ALN for 4 hours (with no pre-treatment).

Figure 3: AF-ALN is internalised by fluid-phase endocytosis in J774 cells and in rabbit osteoclasts. A) J774 cells (i – iii) and rabbit osteoclasts (iv – vi) were incubated with i) 100μM AF-ALN + 1μg/ml wheatgerm agglutinin-633, ii) 100μM AF-ALN + 20μg/ml transferrin-633, iii) 100μM AF-ALN + 250μg/ml TAMRA-dextran, iv) 100μM AF-ALN, v) 250μg/ml TAMRA-dextran, vi) 100μM AF-ALN + TAMRA-dextran + transferrin-633, for 6 hours, then fixed and analysed by confocal microscopy (bar = 10μm). B) J774 cells were incubated with 100μM AF-ALN, 250μg/ml FITC-dextran (FITC-DEX), 10μg/ml wheatgerm agglutinin-633 (WGA-AF633) or 20μg/ml transferrin-633 (TF-AF633) at 4ºC or 37ºC for 1hour. The cells were then washed, incubated for a further 3 hours in treatment-free medium then fixed and analysed by flow cytometry. Results are presented as uptake after incubation at 4ºC as a percentage of the uptake after incubation at 37ºC and are mean (± SEM) of 4 independent experiments. C) J774 cells were incubated for 6 hours with 250μg/ml TAMRA-dextran + 100μM AF-ALN, in the presence or absence of 1mM clodronate, then fixed and analysed by confocal microscopy (bar = 10μm).

Molecular Pharmacology Fast Forward. Published on February 24, 2006 as DOI: 10.1124/mol.105.020776
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 4: Internalisation of alendronate is modified by dansylcadaverine, monensin and bafilomycin A1. J774 cells were incubated for 4 hours with A) 250μM FITC-dextran, B) 100μM AF-ALN, or C) [^{14}C]zoledronate, ± 500μM dansylcadaverine (DC), 20μM monensin (MON) or 50nM bafilomycin A1 (BAF). Cells were then A&B) fixed and analysed by flow cytometry, or C) lysed then internalised [^{14}C]zoledronate quantified by liquid scintillation counting. Results are mean (± SEM) of 4 independent experiments. * = $p<0.05$ compared to treatment with FITC-dextran, AF-ALN or [^{14}C]zoledronate alone. D) J774 cells were incubated for 6 hours with 100μM AF-ALN ± 20μM monensin or 50nM bafilomycin A1, then fixed and analysed by confocal microscopy (bar = 10μm).

Figure 5: Dansylcadaverine, monensin and bafilomycin A1 prevent the inhibitory effect of alendronate or zoledronate on protein prenylation. A) J774 cells or B) rabbit osteoclast-like cells were treated for A) 4 hours or B) 24 hours with 100μM alendronate (ALN) or zoledronate (ZOL), ± 250/500μM dansylcadaverine (DC), 10/20μM monensin (MON) or 25/50nM bafilomycin A1 (BAF). Cell lysates were analysed by western blotting for unprenylated Rap1A and β-actin. Data are representative of 3 independent experiments.

Fig 1

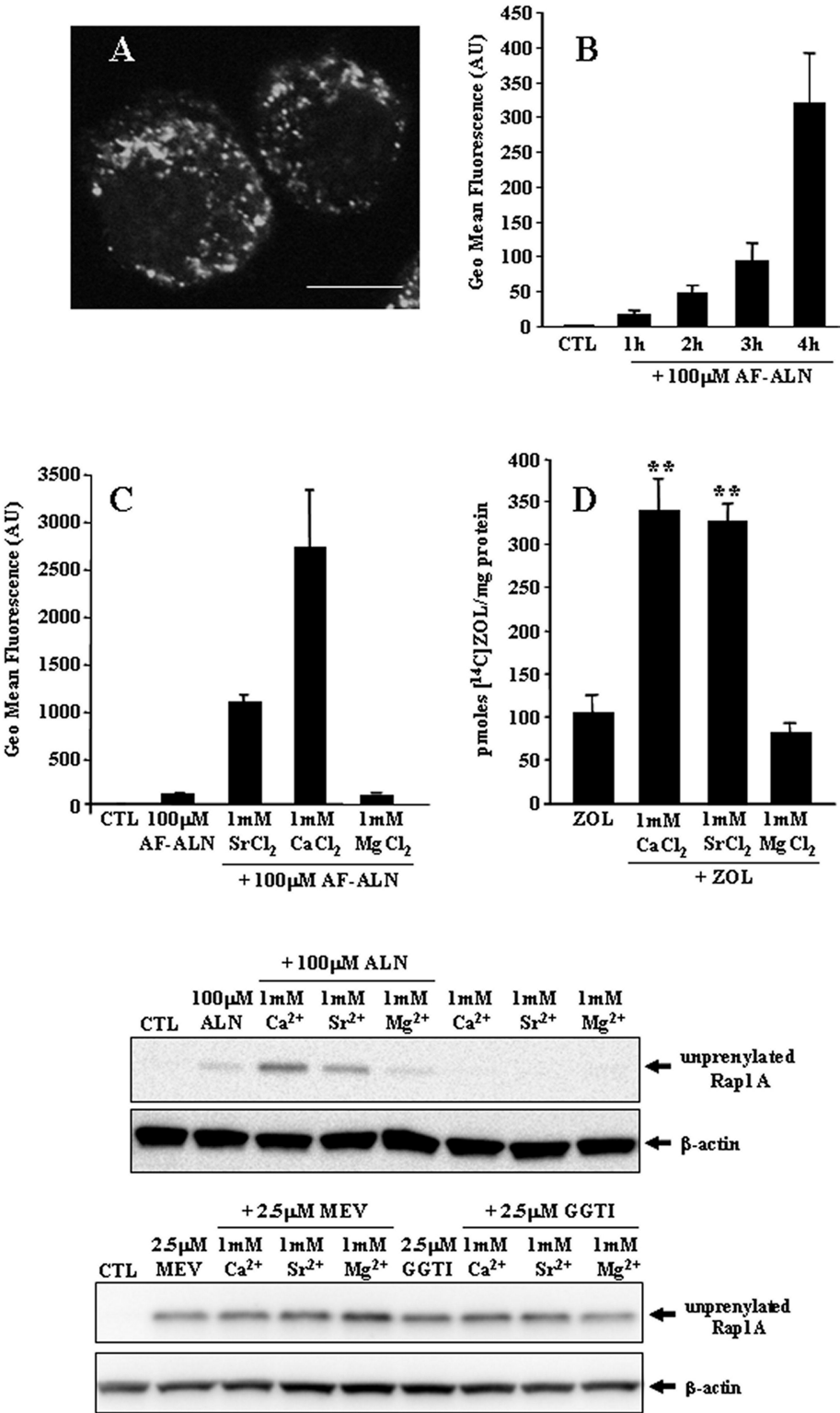
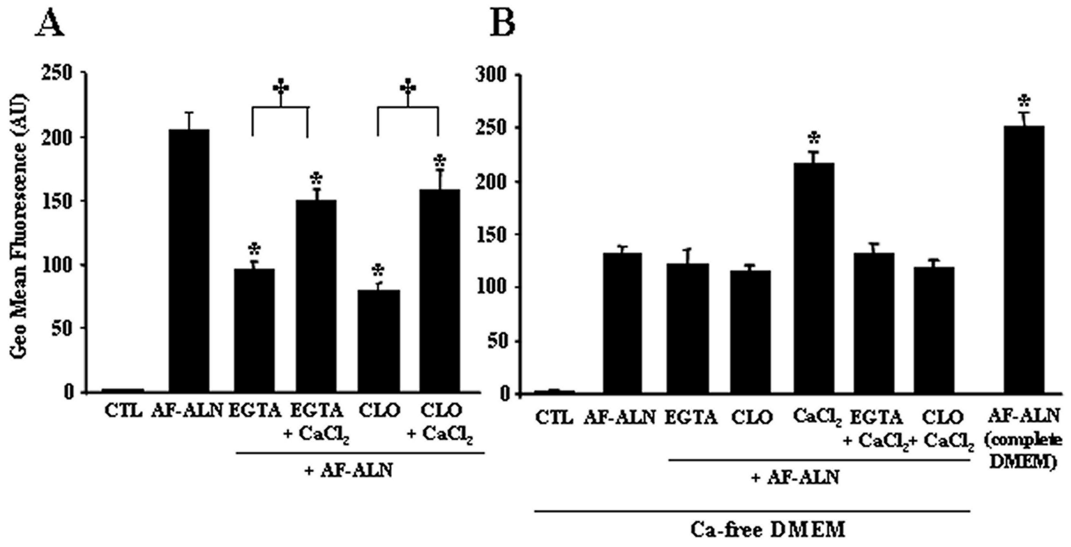
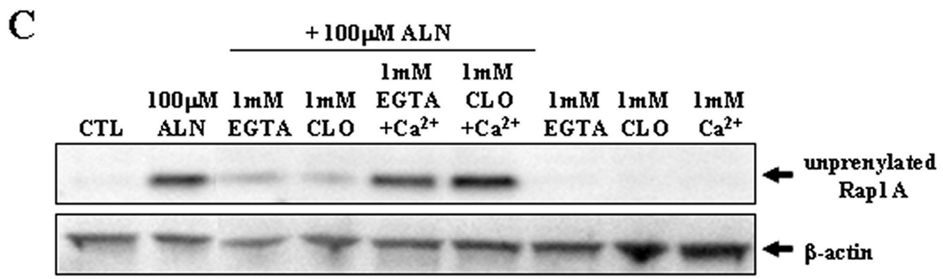

A
B
Geo Mean Fluorescence (AU)
450
400
350
300
250
200
150
100
50
0
CTL
1h
2h
3h
4h
+100μM AF-ALN
C
Geo Mean Fluorescence (AU)
3500
3000
2500
2000
1500
1000
500
0
CTL
100μM AF-ALN
1mM SrCl2
1mM CaCl2
1mM MgCl2
+100μM AF-ALN
D
pmoles [14C]ZOL/mg protein
400
350
300
250
200
150
100
50
0
**
**
ZOL
1mM CaCl2
1mM SrCl2
1mM MgCl2
+ ZOL
E
+100μM ALN
CTL
100μM ALN
1mM Ca2+
1mM Sr2+
1mM Mg2+
1mM Ca2+
1mM Sr2+
1mM Mg2+
unprenylated Rap1A
β-actin
+2.5μM MEV
+2.5μM GGTI
CTL
2.5μM MEV
1mM Ca2+
1mM Sr2+
1mM Mg2+
2.5μM GGTI
1mM Ca2+
1mM Sr2+
1mM Mg2+
unprenylated Rap1A
β-actin

Fig 2


A
B
Geo Mean Fluorescence (AU)
250
200
150
100
50
0
300
250
200
150
100
50
0
CTL
AF-ALN
EGTA
EGTA + CaCl2
CLO
CLO + CaCl2
+ AF-ALN
CTL
AF-ALN
EGTA
CLO
CaCl2
EGTA + CaCl2+ CaCl2
CLO
AF-ALN (complete DMEM)
+ AF-ALN
Ca-free DMEM


C
+100μM ALN
CTL
100μM ALN
1mM EGTA
1mM CLO
1mM EGTA +Ca2+
1mM CLO +Ca2+
1mM EGTA
1mM CLO
1mM Ca2+
unprenylated Rap1A
β-actin

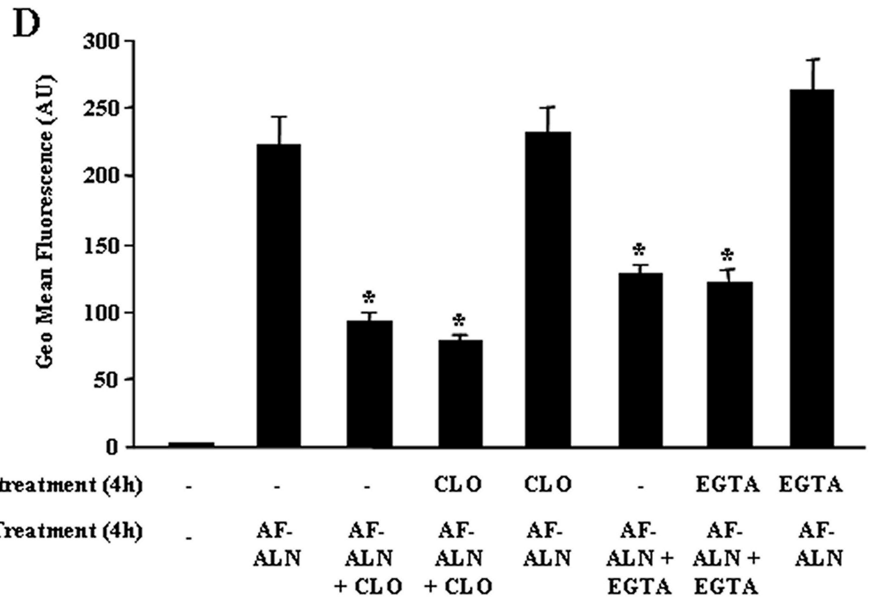
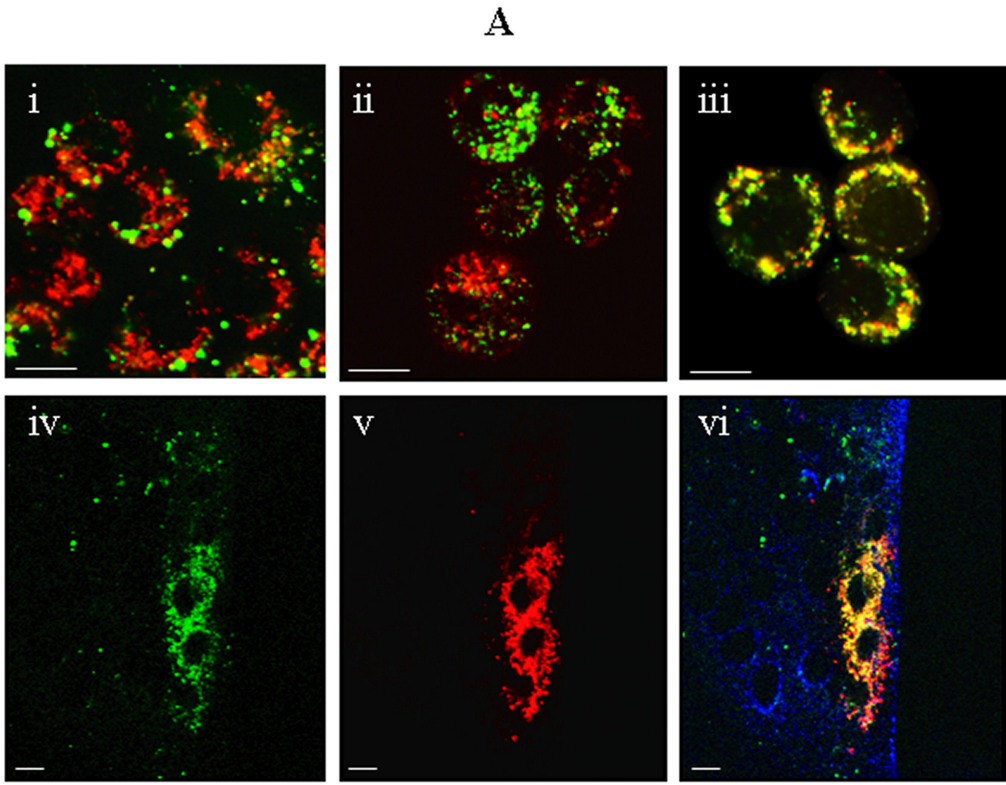
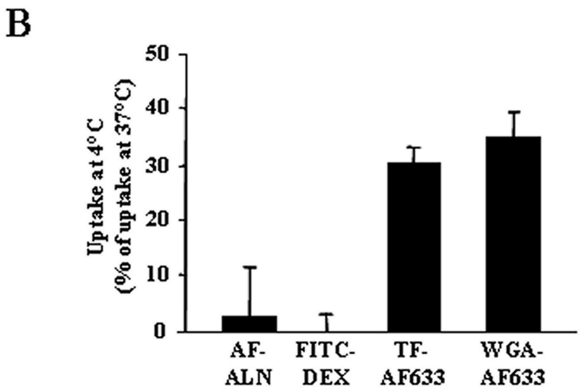
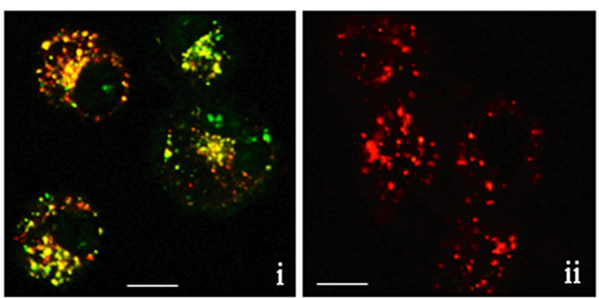
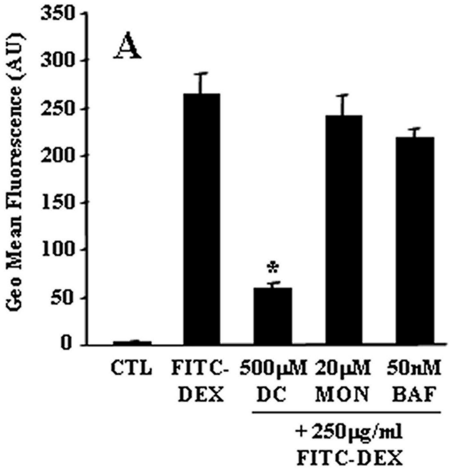

D
Geo Mean Fluorescence (AU)
300
250
200
150
100
50
0
Pretreatment (4h)
Treatment (4h)
-
AF-ALN
AF-ALN + CLO
CLO
AF-ALN + CLO
CLO
AF-ALN
-
AF-ALN + EGTA
EGTA
AF-ALN + EGTA
EGTA
AF-ALN

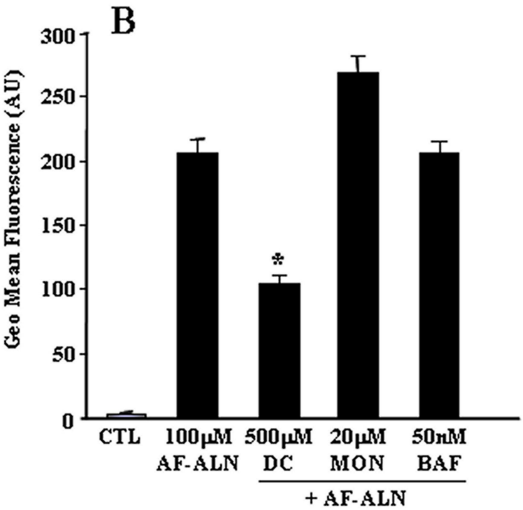
Fig 3

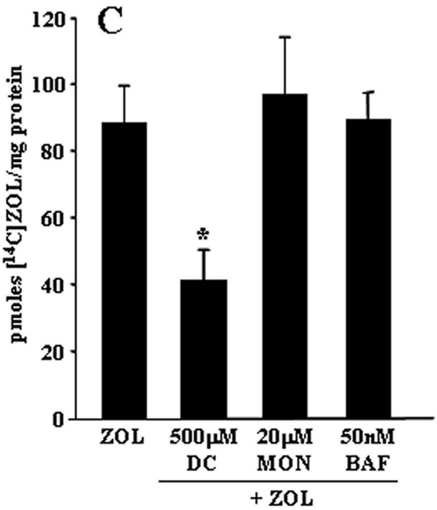

A
i
ii
iii
iv
v
vi


B
Uptake at 4°C
(% of uptake at 37°C)
50
40
30
20
10
0
AF-
ALN
FITC-
DEX
TF-
AF633
WGA-
AF633


C
i
ii


A
Geo Mean Fluorescence (AU)
350
300
250
200
150
100
50
0
*
CTL
FITC-DEX
500μM DC
20μM MON
50nM BAF
+250μg/ml FITC-DEX


B
Geo Mean Fluorescence (AU)
300
250
200
150
100
50
0
*
CTL
100μM AF-ALN
500μM DC
20μM MON
50nM BAF
+ AF-ALN


C
pmoles [14C]ZOL/mg protein
120
100
80
60
40
20
0
*
ZOL
500μM DC
20μM MON
50nM BAF
+ ZOL

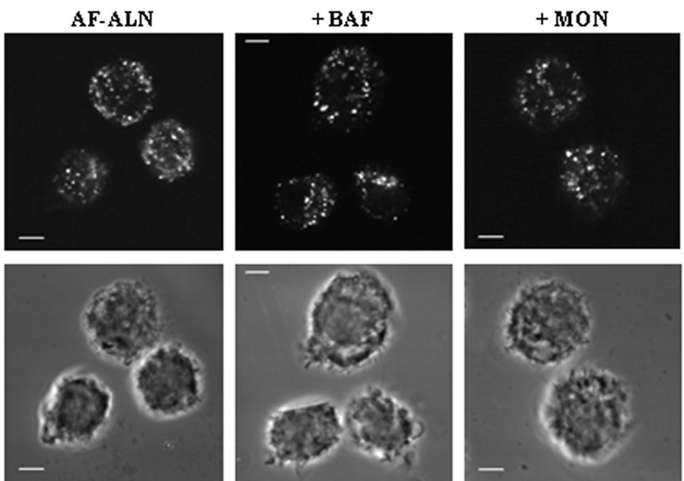
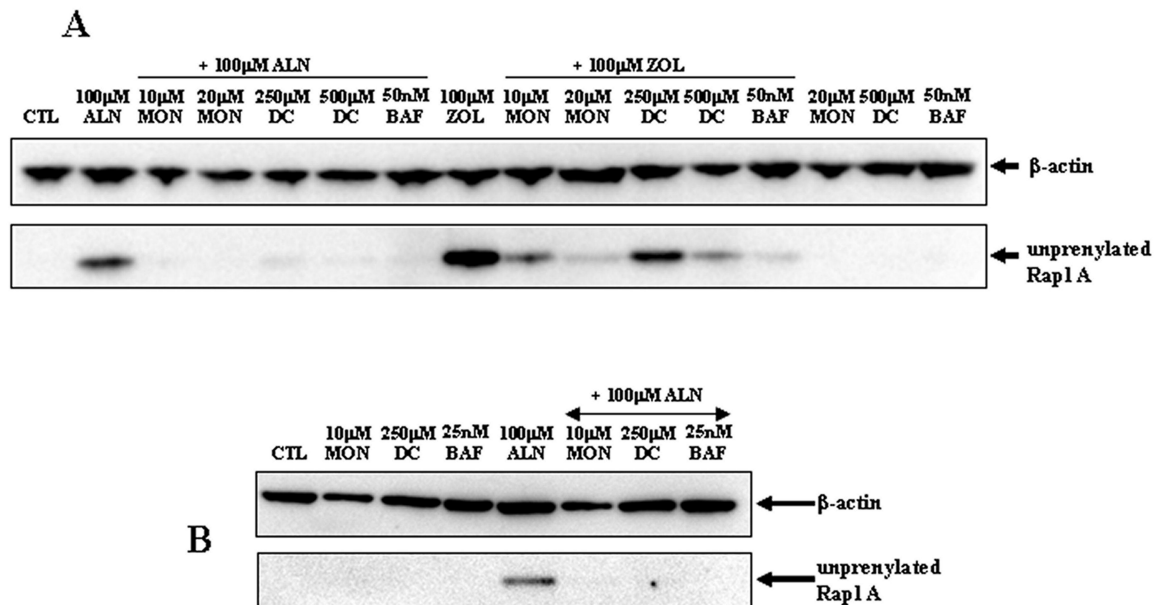

D
AF-ALN
+BAF
+MON

Fig 5


A
+ 100μM ALN
+ 100μM ZOL
CTL
100μM ALN
10μM MON
20μM MON
250μM DC
500μM DC
50nM BAF
100μM ZOL
10μM MON
20μM MON
250μM DC
500μM DC
50nM BAF
20μM MON
500μM DC
50nM BAF
β-actin
unprenylated Rap1A
B
+ 100μM ALN
CTL
10μM MON
250μM DC
25nM BAF
100μM ALN
10μM MON
250μM DC
25nM BAF
β-actin
unprenylated Rap1A