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Profiling signalling pathways of the receptor activator of NF-κB ligand-induced osteoclast formation in mouse monocyte cells, RAW264.7

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Summary. Cell-based signal chemical genomics can profile the signalling pathway for certain cellular events by using a target-known chemical library. To ascertain its usefulness, the receptor activator of NF-κB ligand (RANKL)-induced osteoclastogenesis in mouse monocyte/macrophage cells RAW264.7 was used as an in vitro experimental model. Of 180 target-known inhibitors/activators formatted in a 384-well plate, 8 chemicals were shown to inhibit the osteoclast formation, but 4 chemicals enhanced this process. A variety of references support, or possibly lead one to expect the effects of these 12 chemicals on the cellular process of osteoclastogenesis in RAW264.7 cells, but several signalling pathways were newly found in this study; for example, CA-074 Me inhibiting cathepsin B and nitrendipine blocking the calcium channel could have the potential to inhibit the osteoclast formation as well as bone resorption. This is a simple but very fast and powerful method of profiling the signalling pathway of certain cellular events. Signal chemical genomics could provide invaluable information for the exploration of new target signalling processes and further target-based drug discovery strategies.

Keywords: Drug discovery – Osteoclastogenesis – RAW264.7 cells – Receptor activator of NF-κB ligand (RANKL) – Signal chemical genomics

Introduction

Chemical genomics is an emerging field, integrating the latest developments in tools and technologies from a variety of disciplines, such as combinatorial chemistry, informatics, synthetic chemistry, cell-based assays, microarrays, genomics, bioinformatics, toxicogenomics, and proteomics. In drug discovery and development, chemical genomics aims to accelerate the process and to avoid late-stage failure (Caron, 2004).

From the outset of the drug development process, it is critical to understand what is going to be targeted in the disease and what effect it may have not only on the diseased tissue, but also on the quality of the patient's life. Therefore, a tremendous effort to understand the complex biological situation of diseases is required and this understanding could be a promising way to speed up and further industrialize target-based drug discovery (Russel and Michne, 2004).

To identify a target molecule, the first step is to discover the signal transduction pathway contributing to the disease state. The discovery of signalling pathways and further mapping of the key signalling molecules in biochemical pathways have not only transformed our understanding of how cells operate, but also had an enormous impact on the development of new therapeutic approaches for the treatment of nearly every human disease (Persidis, 1998). However, communication among signalling pathways involved in diseases is too sophisticated for the pathophysiological network of signals to be easily deciphered.

To simplify the process for predicting and/or finding the signal transduction pathway, we propose here the cell-based signal chemical genomics approach using a target-known chemical library. In this study, the receptor activator of NF-κB ligand (RANKL)-induced osteoclastogenesis in mouse macrophage cells RAW264.7 was used as an in vitro experimental model. RAW264.7 cells have been shown to express RANK and differentiate into tartrate resistance acid phosphatase (TRAP)-positive, functionally multinucleated osteoclasts when cocultured with soluble RANKL (Hsu et al., 1999).

Materials and methods

Chemicals

All chemicals used in this study were randomly selected and purchased from Calbiochem (Germany).

Cell culture

All materials used for cell culture were purchased from HyClone (UT). RAW264.7 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 mg/ml streptomycin with a change of medium every 3 days in a humidified atmosphere of 5% CO_2 at 37 °C.

Osteoclast differentiation assay

RAW264.7 cells were plated in a 384-well plate at the density of 0.5×10^3 cells/well and cultured in α-minimal essential medium (MEM) supplemented with 10% FBS in the presence of 100 ng/ml RANKL (R&D Systems Inc., MN). The following day, 180 chemicals, which were formatted in a 384-well plate at a concentration of 10 mM in dimethyl sulfoxide (DMSO; Sigma, MO), were transferred into cells using QRep 384 Pin Replicator (Genetix, UK), to make a final concentration of around 50 μM. On day 4, multinucleated osteoclasts were visualized by TRAP staining using a leukocyte acid phosphatase kit 387-A (Sigma, MO). The dose-dependent effect of selected chemicals on RANKL-induced osteoclastogenesis was evaluated in a 96-well plate at the density of 1×10^3 cells/well.

TRAP activity assay

The multinucleated cells were fixed with 10% formalin for 10 min and 95% ethanol for 1 min, and then dried. To measure TRAP activity, 25 or 100 μl of citrate buffer (50 mM, pH 4.6) containing 10 mM sodium tartrate and 5 mM *p*-nitrophenylphosphate (Sigma) was added to the dried cell-containing wells of 384-well plates or 96-well plates, respectively. After incubation for 30 min, the enzyme reaction mixtures were transferred into the well of fresh plates containing an equal volume of 0.1 N NaOH. Absorption was measured at 410 nm with Wallac EnVision HTS microplate reader (PerlinElmer, Finland).

Cell proliferation assay

RAW264.7 cells were plated in 96-well plates at the density of 4×10^3 cells/well in α-MEM containing 10% FBS in the presence of 100 ng/ml RANKL. The following day, cells were treated with serially diluted chemicals and incubated for 3 days. RAW264.7 cells in which FBS and RANKL were removed after 1 day were used to show the effect of RANKL-stimulated differentiation on cell proliferation since a temporal arrest in the cell cycle is a prerequisite for cell differentiation. Cell proliferation was then measured with a Cell Counting Kit-8 (Dojindo Molecular Technologies, ML) according to the manufacturer's protocol.

Uniformity validation and statistics

Uniformity for assays using 384-well plate formats was validated by calculating the Z-factor according to Assay Guidance Manual Version 4.1, 2005. Significance was determined by Student's *t*-test and differences were considered significant when $p < 0.05$.

Results

In a 384-well plate, the effect of 180 chemicals on the RANKL-induced osteoclastogenesis in RAW264.7 cells was evaluated (Fig. 1). When homogeneity for assay was calculated according to Assay Guidance Manual Version 4.1, 2005, the Z-factor in a plate uniformity study, which was run over 2 days at 3 plates per day, was 0.62.

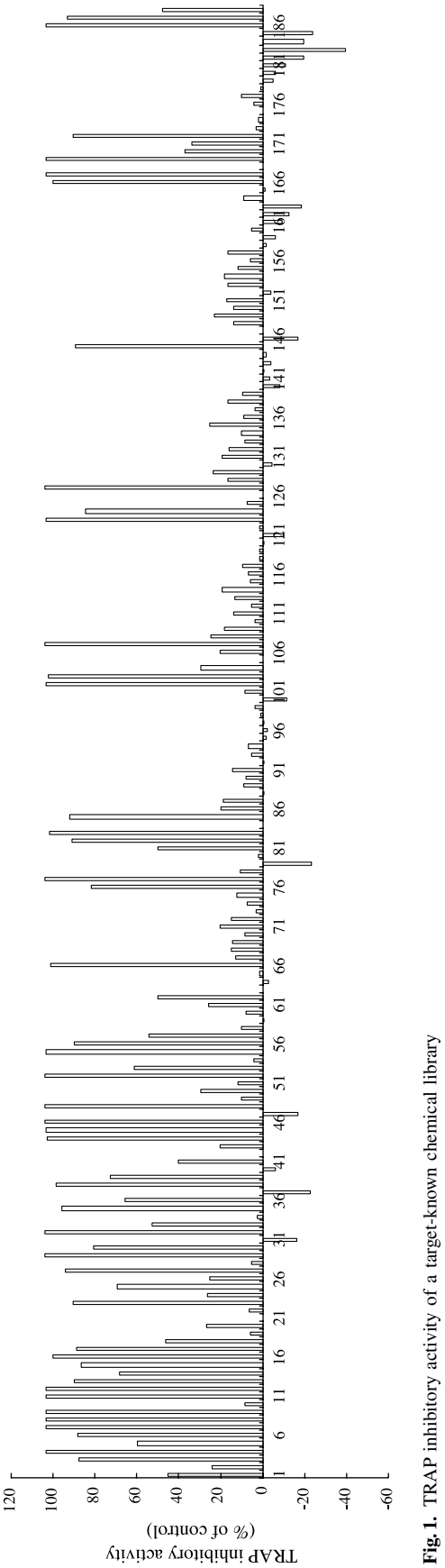

Fig. 1. TRAP inhibitory activity of a target-known chemical library

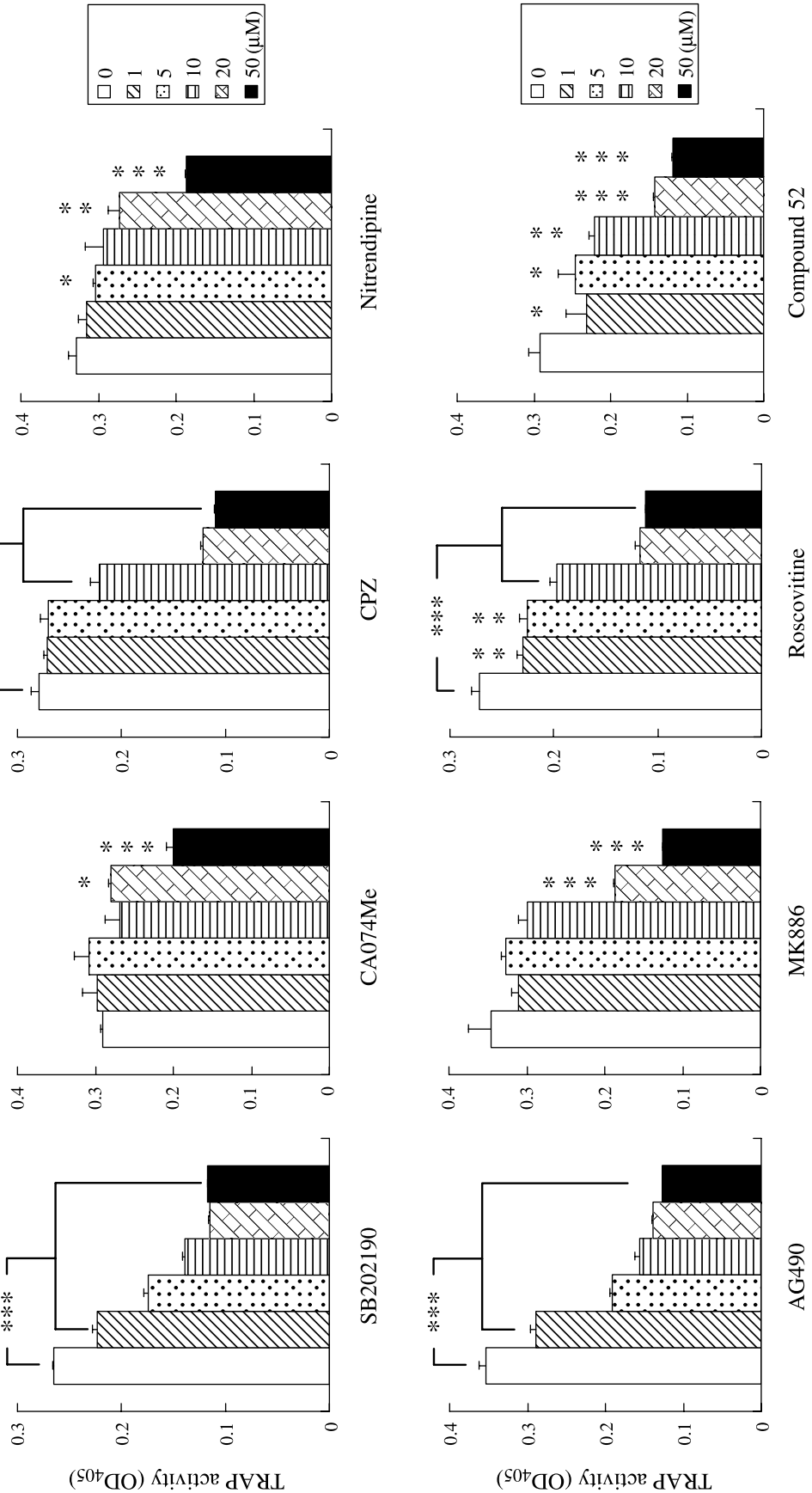

Fig. 2. TRAP activity. The effect of eight chemicals in Table 1 on TRAP activity was evaluated in a 96-well plate. This experiment was performed in triplicate and significance was determined by Student's *t*-test. $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$

Table 1. Chemicals to inhibit the RANKL-induced osteoclastogenesis in RAW264.7 cells

Chemical	Function(s)
SB202190	Inhibits p38 MAP kinase
CA-074 Me	Inhibits intracellular cathepsin B
Chlorpromazine HCl	Inhibits calmodulin-dependent stimulation of cyclic nucleotide phosphodiesterase; Inhibits NOS; Inhibits TNF-α production; Inhibits inward rectifier K^+ current
Nitrendipine	Blocks L-type voltage-sensitive calcium channel
AG490	Inhibits EGFR tyrosine kinase
MK886	Inhibits leukotrienes biosynthesis by preventing 5-LOX activation
Roscovitine	A cyclin-dependent kinase inhibitor; Inhibits $p21^{WAF/Cip1}$
Compound 52	Inhibits the cell cycle-regulating kinase Cdc28p and Pho85p kinase

The acceptance criterion is to have a Z-factor ≥ 0.5. In total, 45 chemicals were shown to have >80% of controls in TRAP inhibitory activity. However, in a further step, among these chemicals, 29 with high cytotoxicity were excluded; there were found to be few cells in a plate when observed under a microscope (data not shown). Therefore, 16 chemicals with a potential to inhibit TRAP activity were selected in this experiment.

To ascertain the inhibitory effects of these 16 chemicals, assays in a 96-well plate with various concentrations of chemicals were followed in triplicate. As shown in Fig. 2, 8 out of 16 chemicals significantly inhibited the RANKL-induced TRAP activity in a dose-dependent manner. The functions of these chemicals are summarized in Table 1 and the inhibitory effects of these chemicals on TRAP-stained osteoclast formation are shown in Fig. 3. The effect of these chemicals on cell proliferation (or viability) was also evaluated in order to prove that the inhibitory effect of these chemicals on the osteoclast formation was not due to the cytotoxicity. Five chemicals (AG490, Compound 52, CPZ, MK886, and roscovitine) were shown to attenuate the rate of RAW264.7 cell proliferation in the process of RANKL-induced osteoclast formation at high concentrations (20–50 μM), but no cytotoxicity was observed in this experiment (data not shown).

In the experiment using a 384-well plate, 5 chemicals were shown to increase the TRAP activity by >15% compared with that in RANKL-treated controls. Although FK506 increased TRAP activity, there were no multinucleated cells in the plate when observed under a microscope, so it was therefore excluded in this step (date not shown). The functions of 4 out of 5 chemicals are summarized in Table 2. The activities of 4 chemicals that enhance the osteoclast formation were also evaluated in a 96-well plate (Fig. 4). Herbimycin A significantly enhanced the TRAP activity at 50 μM, and dexamethasone, NS-398, and RS-13022 significantly enhanced that at 25 μM.

Discussion

Major biotechnology and pharmaceutical companies are developing and actively carrying out drug discovery programs based on understanding signal transduction pathways (Persidis, 1998). Cellular signalling pathways integrate the actions of ligands, substrates and proteins that comprise networks of forward signals, feedback loops and modulatory actions. Defining the signalling cascades of living systems is a major challenge in cell biology and methods of conducting experiments that provide such information are extremely desirable. Therefore, chemically-driven strategies such as signal chemical genomics can provide a powerful method of identifying numerous pathways in living cells.

Here, the RANKL-induced osteoclast formation in RAW264.7 cells was used as an experimental model to show the possibility of predicting or finding the cellular process-related signalling pathway. When the effect of 180 target-known inhibitors/activators on the RANKL-induced osteoclastogenesis was evaluated, 8 chemicals were shown to inhibit this cellular process. Among these chemicals, SB202190 is well-known to inhibit the osteoclastogenesis. Inhibition of p38 mitogen-activated protein (MAP) kinase by SB202190 resulted in a strong suppression in the exogenous RANKL-dependent mouse bone marrow and bone resident precursor cell cultures (Lee et al., 2002).

CA-074 Me, a cathepsin B inhibitor, dose-dependently inhibited the resorptive activity of isolated rat osteoclasts cultured on bone slices with a maximal effect at 50 μM. In addition, it inhibited bone resorption in vitro when administered subcutaneously at a dose of 60 μg/g body weight (Buttle et al., 1992; Hill et al., 1994). Several cathepsins, such as cathepsin C, D, B, E, G and L, were initially demonstrated to take part in the degradation of organic bone matrix in osteoclasts, and cathepsin K, which has highly proteolytic activity and is localized primarily in osteoclasts, was discovered in 1995. Each cathepsin is specifically localized in the osteoclast and it implies the cooperative contribution of each cathepsin to the process of osteoclastic bone resorption. Recently, since cathepsin K plays a critical role in the degradation of bone and appears to be a limiting step in osteoclastic bone resorp-

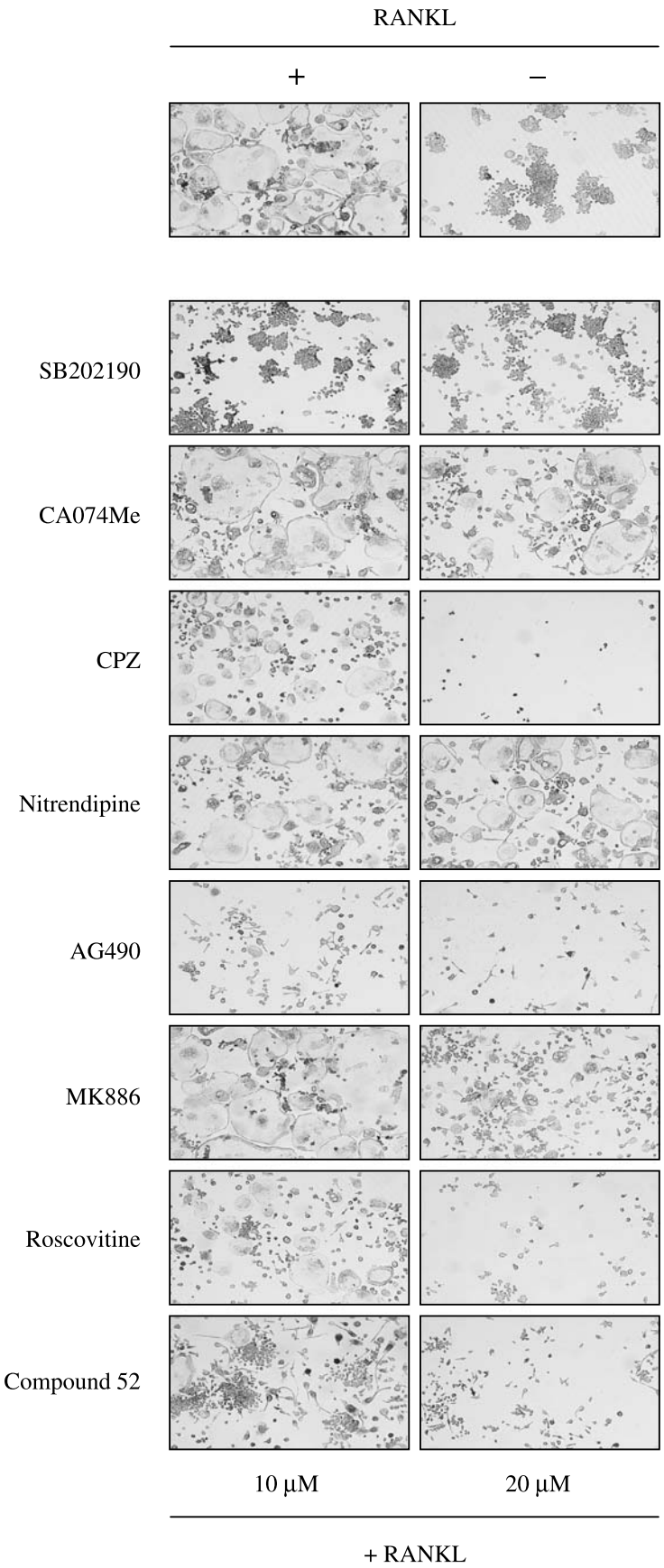

Fig. 3. TRAP staining. Cells were stained for TRAP and photographed

Table 2. Chemicals to enhance the RANKL-induced osteoclastogenesis in RAW264.7 cells

Chemical	Function(s)
Herbimycin A	Inhibits Hsp90; Inhibits c-Src related bone resorption
Dexamethasone	A synthetic glucocorticoid analog
NS-398	Inhibits COX-2
RG-13022	Inhibits EGFR tyrosine kinase

tion, cathepsin K has been attractive target for therapeutic intervention to prevent and ameliorate the significant deleterious impact of osteoporosis. Here, we report for the first time that CA-074 Me as a cathepsin B inhibitor could dose-dependently inhibit the osteoclast formation as well as the resorptive activity of osteoclasts, but further investigations are required to ascertain which signalling pathways could regulate the inhibitory activity of CA-074 in osteoclast formation.

Chlorpromazine (CPZ) inhibited in vitro bone resorption (Hall et al., 1996). The functional action of CPZ on the osteoclastogenesis can be described from several viewpoints: 1) Calmodulin antagonists such as trifluoperazine are structurally related to CPZ and they have been shown to inhibit osteoclast formation in a dose-dependent manner (Zhang et al., 2003). Since calmodulin plays an important role in regulating the function of mature osteoclasts, the inhibitory action of CPZ on osteoclasts might be via calmodulin signalling. 2) CPZ also inhibits nitric oxide synthase (NOS) activity that could positively affect the osteoclastic differentiation (Palacios et al., 1993; Nicolin et al., 2005). This suggests that the NOS signalling pathway can not be excluded from the explanation of the inhibitory effect of CPZ on the osteoclast formation. 3) Tumor necrosis factor (TNF)-α blockade may also account for the cellular mechanism of CPZ. Via TNF-α blockade, CPZ can reduce bone loss in experimental periodontitis (de Lima et al., 2000) and it prevents lethality in endotoxic shock due to the inhibition of TNF-α synthesis as well as to the interference with signalling pathways triggered by TNF-α coupling with its receptors (Bleeker et al., 1999). TNF-α has been reported to stimulate osteoclast differentiation and bone resorption by osteoclasts (Azuma et al., 2000). 4) The ability of CPZ to inhibit inward-rectifying K^+ channels in myocytes may be of relevance to the inhibition of osteoclasts by CPZ, since the presence of such channels has been demonstrated in osteoclasts (Ravesloot et al., 1989; Kon et al., 1994). It has been suggested that the inhibition of inward rectifier K^+ current causes membrane depolarization, the elevation of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and the inhibition of osteoclastic bone resorption (Okamoto et al., 2001).

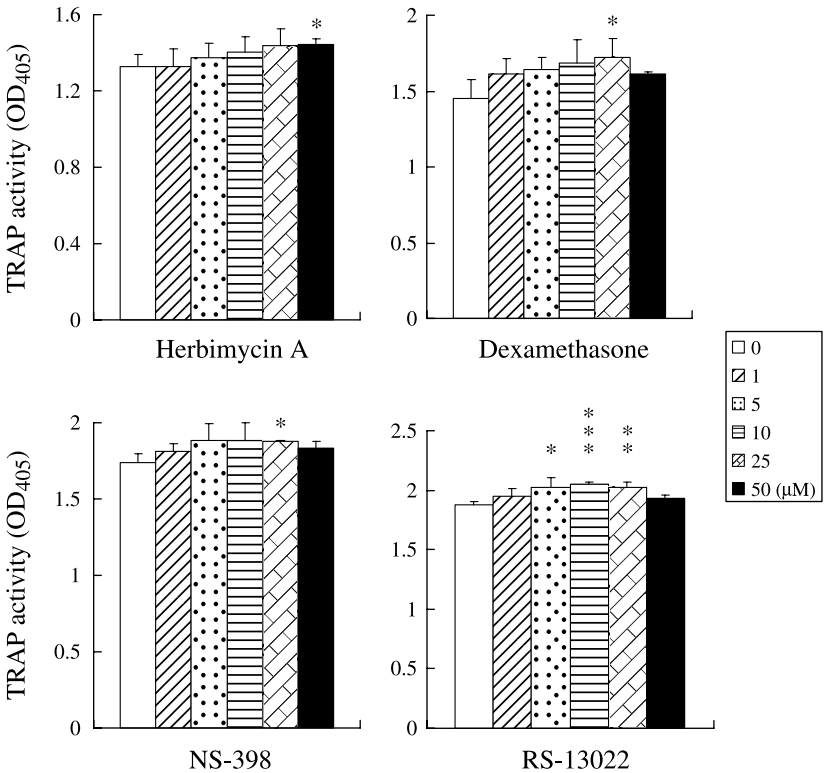

Fig. 4. TRAP activity. The effect of four chemicals in Table 2 was evaluated in a 96-well plate. This experiment was performed in triplicate and significance was determined by Student's *t*-test. $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$

As well as K^+ channels, the calcium channel is also an important factor in osteoclastogenesis and the discovery of new calcium antagonists is one of the drug-development processes for osteoporosis. The increase of $[Ca^{2+}]_i$ by calcium channel antagonists can lead to a decrease in bone resorption (Ritchie et al., 1994). Nitrendipine, a specific L-type voltage-sensitive calcium channel blocker, has been shown to inhibit TNF-stimulated resorption in the fetal rat limb bones (Shankar and Stern, 1993). Considering our data, the calcium channel blocker could affect the process of osteoclast formation as well as bone resorption.

Epidermal growth factor receptor (EGFR) is expressed by osteoclasts (Wang et al., 2004). Interestingly, EGFR ligands have been found to stimulate osteoclastic bone resorption in vitro and in vitro in bone organ culture (Raisz et al., 1980; Marie et al., 1990), and the inhibition of EGFR tyrosine kinase activity by AG1478 (EGFR tyrosine kinase inhibitor) decreased the generation of osteoclasts from cultured bone marrow cells (Wang et al., 2004). The inhibitory effect of AG490, a potent inhibitor of EGFR tyrosine kinase, on the RANKL-induced osteoclastogenesis could stem from its potential to inhibit EGFR tyrosine kinase activity in RAW264.7 cells that results in the decrease of osteoclast differentiation from its precursors.

The leukotrienes and peptide-leukotrienes are 5-lipoxygenase (5-LOX) metabolites of arachidonic acid (AA). Application of exogenous leukotrienes results in increased osteoclast formation, bone resorption, and inhibited bone formation (Garcia et al., 1996; Traianedes et al., 1998). These studies provide evidence of the inhibitory effect of MK886, an inhibitor of 5-LOX, on the osteoclast formation.

Roscovitine, a cyclin-dependent kinase inhibitor, has been reported to dose-dependently decrease the activity of RUNX2, which promotes preosteoblast growth and osteoblast lineage commitment (Qiao et al., 2006), but its effect on the osteoclastogenesis has not been studied. However, taking into consideration the report showing the association of osteoclast differentiation with transient up-regulation of $p21^{WAF/Cip1}$ (Okahashi et al., 2001) and the activity of roscovitine in down-regulating $p21^{WAF/Cip1}$ (Wartenberg et al., 2002), the inhibitory effect of roscovitine on the osteoclast formation might result from the blockade of the $p21^{WAF/Cip1}$ signalling pathway.

The involvement of cell cycle-regulating kinase in the process of osteoclast differentiation maybe also explain the inhibitory effect on the osteoclast formation of compound 52, which can inhibit the cell cycle-regulating kinase Cdc28p and Pho85p kinase (Gray et al., 1998), although its function is not clear.

On the other hand, chemicals that have been-well known to inhibit certain types of signal transduction were shown to enhance osteoclastogenesis. In this study, 5 chemicals (herbimycin A, dexamethasone, NS-398, RG-13022 and FK506) were shown to enhance the osteoclast formation in a 384-well plate, but the effect of FK506 was not confirmed in a 96-well plate.

The heat shock protein 90 (Hsp90) inhibitor, herbimycin A, enhanced osteoclastogenesis in RANKL/M-CSF (macrophage colony stimulating factor)-treated bone marrow macrophage (Price et al., 2005). In addition, other Hsp90 inhibitors such as 17-allylamino-17-demethoxygeldanamycin and radicicol also enhanced osteoclastogenesis. These data are consistent with our observation, but conversely, it has been reported that herbimycin A inhibited the formation of bone resorbing osteoclasts in mouse long-term marrow cultures by irreversibly and directly inhibiting $pp60^{c-src}$ tyrosine kinase (Yoneda et al., 1993).

In histomorphometric studies in glucocorticoid-induced osteoporosis, an increment in the number of osteoclasts and bone resorbing sites was observed. Dexamethasone, a synthetic glucocorticoid analog, also stimulated osteoclast-like cell formation, but at high concentration, depressed it (Shuto et al., 1994; Takuma et al., 2003). The down-regulation of inhibitory cytokines such as interferon-β (IFN-β) by dexamethasone may allow the osteoclast progenitors to be freed from the suppression of osteoclastogenesis (Takuma et al., 2003).

Prostaglandin H_2 (PGH_2) is produced from AA by prostaglandin H syntheses (PGHSs; also termed cyclooxygenase [COX]) and it is then converted by several terminal syntheses to the major active prostanoids produced in vitro such as PGD_2 and PGE_2. The significance of COX-2 in inflammation is highlighted by the observation that COX-2 inhibitors block the synthesis of PGE_2. In this study, NS-398, a specific inhibitor of COX-2, was shown to slightly enhance osteoclastogenesis. This was consistent with the report showing that NS-398 enhanced osteoclast formation induced by the parathyroid hormone in the co-culture system (Take et al., 2005). However, conversely, RANKL selectively induces COX-2 expression, that results, in turn, in the production of PGE_2 in RAW264.7 cells (Han et al., 2005). In addition, the blockade of COX-2 by celecoxib inhibited the differentiation of BMMs into TRAP-positive osteoclastic cells, and this inhibition was reversed by the addition of exogenous PGE_2. The relationship between AA metabolism and osteoclastogenesis is still controversial and not conclusive. However, here, to explain the activity of NS-398 in enhancing the osteoclast formation, the possibility of the shunt of AA

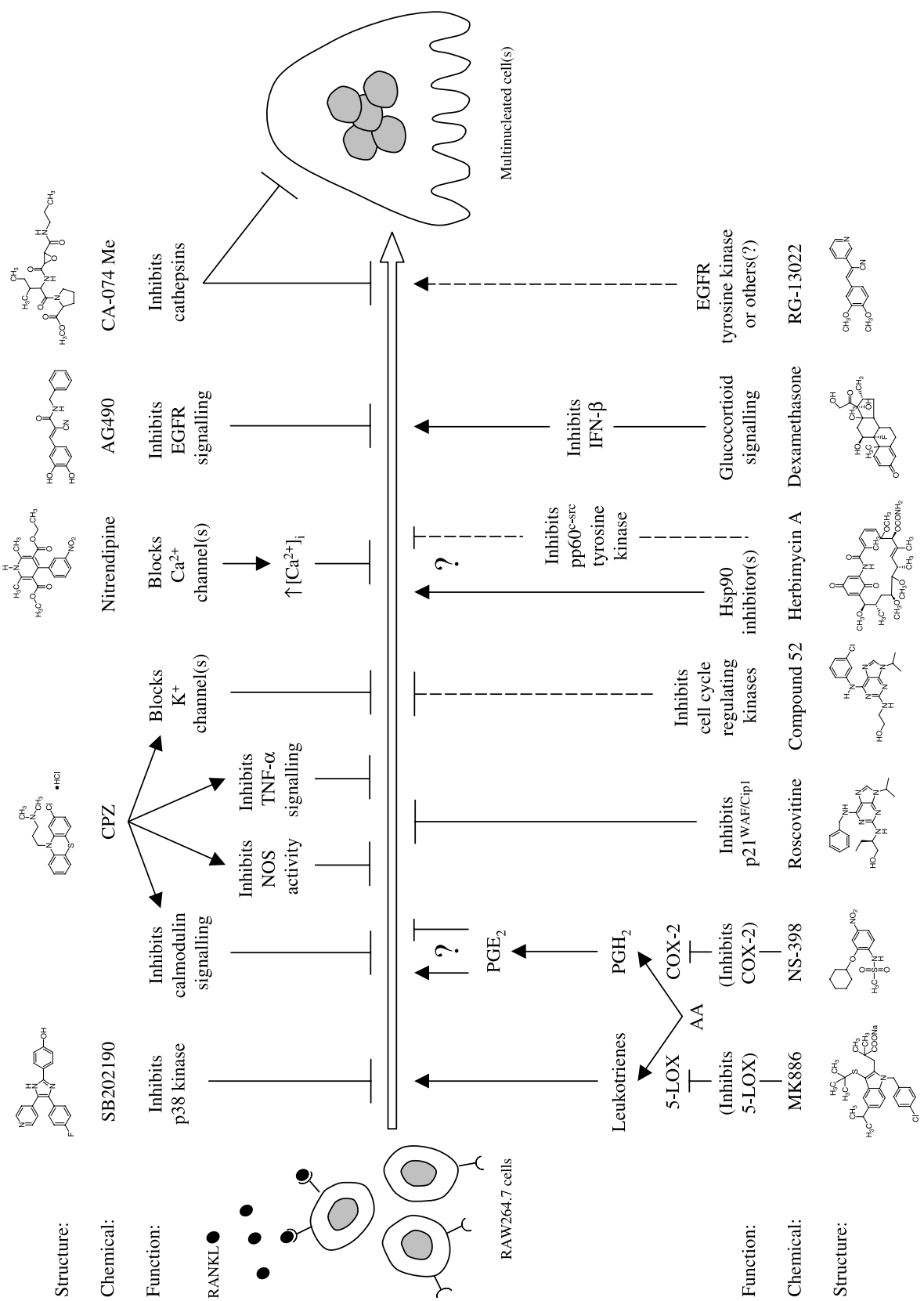

Fig. 5. Signalling pathways involved in RANKL-induced osteoclastogenesis in RAW264.7 cells

metabolism from the COX to the LOX pathway must be considered.

As well as AG1478 and AG490, RG-13022 is a selective EGFR tyrosine kinase inhibitor, but it was shown to slightly enhance the osteoclast formation. In cells, AG490 and RG-13022 might act in a similar way by inhibiting EGFR tyrosine kinase, but controversial results were obtained in this study. However, considering the fact that the inhibitory effect of AG490 on the osteoclast formation was shown to follow the inhibition of RAW264.7 cell proliferation and the activation by RG-13022 with no effect on cell proliferation, the subsequent cellular mechanism or action of both chemicals after inhibiting EGRG tyrosine kinase might be quite different. Additionally, the possibility that other different target proteins for these chemicals are present and involved in the process of osteoclastogenesis should not be excluded.

FK506, which is used today as an immunosuppressant drug in organ transplantation, has been reported to induce osteoporosis by action on osteoclasts, promoting the mRNA expression of the osteoclast differentiation factor and osteoclast differentiation and maturation (Fukunaga et al., 2004). However, when 0.15–2.5 mM FK506 was treated in RAW264.7 cells, it had no effect on cell proliferation or TRAP activity, but it inhibited the formation of TRAP-positive multinucleated cells at 1.2 mM (Shui et al., 2002). In this study, FK506 was shown to induce TRAP activity at $<60\ \mu M$, but its activity resulted from the formation of TRAP-positive mononucleated cells, not multinucleated cells. More experiments to elucidate the effect of FK506 on osteoclasts are required.

Using a signal chemical genomics approach, signalling pathways involved in the RANKL-induced osteoclastogenesis of RAW264.7 cells can be summarized as in Fig. 5. As described above, most of the mechanisms can be fully supported by, or possibly expected from a variety of references, but several signalling pathways were newly found in this study; for example, CA-074 Me inhibiting cathepsin B and nitrendipine blocking the calcium channel could have the potential to inhibit the osteoclast formation. However, there are also several controversial reports in the explanation of the mechanism of osteoclastogenesis, suggesting that the signalling pathway could be different depending on a variety of factors, such as the origin, type, or culture method of used cells, used stimulants and so on. Because of this, the target molecule decided to be used in a specific in vitro model might not be applied to another in vitro model. In conclusion, in the first step of drug discovery and development, a signal chemical genomics approach could be useful in deciding the appropriate target molecule (or signalling pathway) in certain in vitro experiment models. Also required is the continued evolution of the accuracy and versatility of this approach, but it can make us profile the pathophysiological network of signals in a certain cellular event simply and quickly, and this finding could provide invaluable information for the exploration of the new target signalling and further target-based drug discovery strategies.

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