BONE BIOLOGY

Complete abrogation of key osteoclast markers with a membrane-anchored tissue inhibitor of metalloproteinase

A NOVEL APPROACH IN THE PREVENTION OF OSTEOCLASTOGENESIS

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Aims
Tissue inhibitors of metalloproteinases (TIMPs) are the endogenous inhibitors of the zinc-dependent matrix metalloproteinases (MMP) and A disintegrin and metalloproteinases (ADAM) involved in extracellular matrix modulation. The present study aims to develop the TIMPs as biologics for osteoclast-related disorders.

Methods
We examine the inhibitory effect of a high affinity, glycosyl-phosphatidylinositol-anchored TIMP variant named ‘T1 Prα TACE’ on receptor activator of nuclear factor kappa-B ligand (RANKL)-induced osteoclast differentiation.

Results
Osteoclast progenitor cells transduced with T1 Prα TACE failed to form tartrate-resistant acid phosphatase (TRAP)-positive osteoclasts or exhibit bone-resorbing activity following treatment with RANKL. At the messenger RNA level, T1 Prα TACE strongly attenuated expression of key osteoclast marker genes that included TRAP, cathepsin K, osteoclast stimulatory transmembrane protein (OC-STAMP), dendritic cell-specific transmembrane protein (DC-STAMP), osteoclast-associated receptor (OSCAR), and ATPase H+-transporting V0 subunit d2 (ATP6V0D2) by blocking autoamplification of nuclear factor of activated T cells 1 (NFATc1), the osteoclastogenic transcription factor. T1 Prα TACE selectively extended p44/42 mitogen-activated protein kinase activation, an action that may have interrupted terminal differentiation of osteoclasts. Inhibition studies with broad-spectrum hydroxamate inhibitors confirmed that the anti-resorptive activity of T1 Prα TACE was not reliant on its metalloproteinase-inhibitory activity.

Conclusion
T1 Prα TACE disrupts the RANKL-NFATc1 signalling pathway, which leads to osteoclast dysfunction. As a novel candidate in the prevention of osteoclastogenesis, the TIMP could potentially be developed for the treatment of osteoclast-related disorders such as osteoporosis.

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Article focus
- We aim to develop a tissue inhibitor of metalloproteinases (TIMP) for osteoclast inhibition.
- ‘T1 Prα TACE’ is a glycosyl-phosphatidylinositol-anchored TIMP capable of blocking osteoclastogenesis.

Key messages
- T1 Prα TACE inhibits osteoclastogenesis in osteoclast progenitor cells.
- T1 Prα TACE attenuates transcription of multiple key osteoclast markers.
T1\textsuperscript{ProtACE} blocks autoamplification of master transcription factor nuclear factor of activated T cells.\textsuperscript{1} 

**Strengths and limitations**

- This is a novel antosteoclastogenesis strategy that has never been attempted before.
- A broad range of cell- and molecular-based approaches are used to delineate the activities of T1\textsuperscript{ProtACE}.
- Animal studies are required to assess the potency of the TIMP.

**Introduction**

Bone homeostasis is a complex balancing act maintained by the coordinated activities of bone-forming osteoblasts and bone-resorbing osteoclasts. Derived from monocytic/macrophagic lineage, osteoclasts are giant multinucleated cells uniquely designed for the dissolution and absorption of bone. During bone remodelling, osteoclast progenitor cells are recruited by osteoblasts to the site of remodelling, wherein macrophage-colony stimulating factor (M-CSF) and receptor activator of nuclear factor-kB (NF-κB) ligand (RANKL) are secreted to initiate osteoclast differentiation.\textsuperscript{1} Binding of M-CSF and RANKL to their cognate receptors, colony-stimulating factor-1 receptor (C-FMS) and receptor activator of nuclear factor kappa-β (RANK), prompted osteoclast progenitor cells to commit to osteoclast lineage-specific growth, a cell fusion event mediated by the dendritic cell-specific transmembrane protein (DC-Stamp).\textsuperscript{2}

To resorb bone effectively, osteoclasts form ring-shaped adhesion structures known as the “sealing zone” with the underlying bone matrix.\textsuperscript{1} It is within the confine of this sealing zone that a number of enzymes, chief among them cathepsin K (CatK), tartrate-resistant acid phosphatase (TRAP), and matrix metalloproteinase (MMP)-9, are secreted to dissolve bone matrix.\textsuperscript{1,3} CatK is one of the most prominent cysteine proteinases expressed in osteoclasts of which the primary function is to degrade bone matrix proteins.\textsuperscript{4-6} Besides its bone-degrading function, CatK also activates the zymogen of MMP-9, a prominent member of the MMP family whose main function is to degrade and remodel the extracellular matrix, including that of bone.\textsuperscript{7} In osteoclasts, MMP-9 is secreted as a latent pro-enzyme that requires activation by CatK in the extracellular space.\textsuperscript{7} Mounting evidence indicates that CatK is indispensable for osteoclast-mediated bone resorption, and targeting CatK could offer a promising strategy to prevent osteoclast-related disorders such as osteoporosis.\textsuperscript{8}

The aim of the current study is to examine the therapeutic potential of tissue inhibitors of metalloproteinases (TIMPs) in the prevention of osteoclast-mediated bone resorption. As the endogenous inhibitors of the MMPs and A disintegrin and metalloproteinases (ADAMs), TIMPs (TIMP-1 to -4) are small proteins of 21 to 27 kDa in molecular mass.\textsuperscript{9} Despite sharing a relatively high degree of sequence homology (40% to 50%), the TIMPs differ vastly in their profiles of metalloproteinase selectivity. For instance, MT1-MMP can be inhibited by TIMP-2, -3, and -4 but not TIMP-1, whereas tumour necrosis factor alpha (TNF-α) converting enzyme (TACE) is selectively inhibited by TIMP-3.\textsuperscript{10,11} A recent study carried out on labral tissue with femoroacetabular impingement (FAI) pathology showed an increased expression of MMP-1 and -2, while the level of TIMP-1 was found to be reduced.\textsuperscript{12}

In this study, we seek to modulate osteoclast formation and function through a membrane-anchored TIMP variant named ‘T1 ProtACE', T1 ProtACE is a glycosylphosphatidylserine (GPI)-anchored TIMP mutant originally engineered for the inhibition of TACE.\textsuperscript{13,14} We show that T1 ProtACE, attenuates RANKL-mediated osteoclast differentiation and bone resorption by preventing nuclear factor of activated T cells (NFATc1) from regulating its own expression. Osteoclast differentiation is fully dependent upon NFATc1,\textsuperscript{15} the expression of which is mediated by the binding of RANKL to its receptor RANK which, in turn, triggers p38 mitogen-activated protein kinase (MAPK) phosphorylation.\textsuperscript{16} As shown in this study, attenuation in NFATc1 expression abrogates the downstream transcription of key osteoclast marker genes that include TRAP, CatK, OC-Stamp, and ATP6VD02. The efficacy of our unique approach may potentially allow for an alternative strategy to be developed for the prevention and treatment of osteoclast-related disorders such as osteoporosis and rheumatoid arthritis.

**Methods**

**Materials.** Unless otherwise stated, all the chemicals and reagents used in this study were supplied by Thermo Fisher Scientific (USA). Antibodies for TIMP-1 (Ab1827), TACE (Ab28233), TRAP (Ab191406), CatK (11239 to 1-AP), and NFATc1 (66963 to 1-lg) were acquired from Abcam (UK), Proteintech (USA), or Novus Biologicals (USA). Osteoclast Identification kit (70-CK20203), TRAP activity test kit (P0332), F-actin staining kit (C2203-S, Actin-Tracker Red-555), RNeasy Mini Kit (74004), GoScript Reverse Transcription System kit (AS001), and GoTaq qPCR Master Mix kit (A6020) were sourced from MultiSciences (China), Beyotime (China), Qiagen (Germany), and Promega (USA). Enzyme-linked immunoassortment assay (ELISA) kit for TNF-α (10602) was the product of Sino Biological (China). Batimastat (BB-94; S7157), ilomastat (galardin, GM6001; S7157), and TNF-α processing inhibitor (TAPI-0; CAS143457-40-3) were the products of Selleckchem (USA) and Santa Cruz Biotechnology (USA), respectively. Bovine cortical dentin slices (0.2 mm) were purchased from Boneslices (Denmark).

**Gelatin zymography, reverse zymography, and ELISA assay kits.** Gelatin zymography and reverse zymography were carried out on 10% polyacrylamide gels essentially as described.\textsuperscript{17} For MMP-2 and -9 detection, the samples were diluted in phosphate-buffered saline (PBS) prior to loading (25 μl/lane) onto acrylamide gels. All the ELISA assays were performed in triplicate in accordance to the manufacturers’ protocols.
Transduction and stable cell line selection. Lentivirus encoding TIMP complementary DNAs (cDNAs) in pLVX vector (Takara, Japan) were transduced in RAW264.7 cells in α-minimum essential medium (α-MEM) supplemented with 8 µg/ml polybrene. For stable cell selection, puromycin (2 µg/ml) was added to the media eight hours post-transduction. To obtain stable cells with the highest fluorescence intensity, the cells were subjected to a second round of selection with fluorescence-activated cell sorting cytometry.

TRAP staining and enzymatic activity assay. To initiate osteoclast formation, RAW264.7 cells in 24-well plates (5,000 cells/well) were induced with RANKL (100 ng/ml) in α-MEM supplemented with 5% fetal bovine serum. At 96 hours following induction, the cells were stained for TRAP and assayed with p-nitrophenyl phosphate (pNPP) for phosphatase activity. TRAP-positive cells with three or more nuclei were identified as osteoclasts. TRAP activity was quantified using a Varioskan colorimetric plate reader (Thermo Fisher Scientific) at 405 nm wavelength. MMP-2, MMP-9, and TNF-α expression were analyzed by gelatin zymography\(^\text{17}\) or ELISA.

F-actin ring assays. Cells were fixed and stained with 4% paraformaldehyde/0.1% phalloidin 120 hours after induction with RANKL essentially as described in the product leaflet.

Osteoclast inhibition study with hydroxamate inhibitors. Batimastat, ilomastat, and TNF alpha processing inhibitor-0 (TAPI-0) were added to RAW264.7 cells from low to high concentrations during induction with RANKL. The cells were cultured for days and stained for TRAP.

Reverse transcription-quantitative polymerase chain reaction. Reverse transcription was performed with the GoTaq qPCR Master Mix kit on a Thermo QuantStudio 5 Real-Time PCR System as follows: initial denaturation at 95°C for five minutes; 40 cycles of denaturation at 95°C for 15 seconds; and annealing/extension of product at 60°C for 60 seconds. Relative gene expression was calculated for phosphatase activity. TRAP-positive cells with three or more nuclei were identified as osteoclasts. TRAP activity was quantified using a Varioskan colorimetric plate reader (Thermo Fisher Scientific) at 405 nm wavelength. MMP-2, MMP-9, and TNF-α expression were analyzed by gelatin zymography\(^\text{17}\) or ELISA.

Results

T1\(\text{TACE}\) suppressed RANKL-mediated osteoclast differentiation and bone resorption. While the majority of the osteoclasts from the control, wild-type TIMP-1, and T1\(\text{TACE}\) groups were > 200 µm in diameter, far fewer osteoclasts were observed in T1\(\text{PrTACE}\) cells (Figure 2a; Supplementary Figure a). Formation of F-actin rings represents a hallmark for osteoclast differentiation.\(^\text{18}\) A complementary staining study performed using phosphoimaging showed a 93% decrease in the number of F-actin rings in T1\(\text{PrTACE}\) cells (Figure 2b, Supplementary Figure a). We next examined if T1\(\text{PrTACE}\) could prevent osteolytic bone resorption on bovine cortical dentin slices. A significant decrease (> 80%) in TRAP staining and area of resorption could be visualized in the cells transduced with T1\(\text{PrTACE}\), but not wild-type TIMP-1 (p < 0.001, paired t-test) (Figures 3a and 3b).

Bone resorption pit assay. RAW264.7 cells (3 × 10^5 cells per well) transduced with wild-type TIMP-1 or T1\(\text{PrTACE}\) were seeded on bovine cortical dentin slices and incubated for 21 days in α-MEM supplemented with 100 ng/ml RANKL (n = 4). Following gentle sonication and brushing, the bone slices were stained with 1% toluidine blue before images were taken and quantified for bone resorption using ImageJ software (USA).

Statistical analysis. Statistical analysis between subgroups was carried out with the paired t-test or analysis of variance (ANOVA) using Social Science Statistics. All data are presented as the means of three experimental repeats and standard deviation (SD). Only p-values < 0.05 were considered as statistically significant. To ensure the reproducibility of the results, all the experiments that required quantification had been performed on at least three biological replicates for a minimum of three times.

F-actin ring analyses. Cells were fixed and stained with 4% paraformaldehyde/0.1% phalloidin 120 hours after induction with RANKL essentially as described in the product leaflet.

Osteoclast inhibition study with hydroxamate inhibitors. Batimastat, ilomastat, and TNF alpha processing inhibitor-0 (TAPI-0) were added to RAW264.7 cells from low to high concentrations during induction with RANKL. The cells were cultured for days and stained for TRAP.

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Bone resorption pit assay. RAW264.7 cells (3 × 10^5 cells per well) transduced with wild-type TIMP-1 or T1\(\text{PrTACE}\) were seeded on bovine cortical dentin slices and incubated for 21 days in α-MEM supplemented with 100 ng/ml RANKL (n = 4). Following gentle sonication and brushing,
T1Pr\textsuperscript{α}TACE: a high affinity, glycosylphosphatidylinositol (GPI)-tethered tissue inhibitor of metalloproteinases (TIMP) engineered for the inhibition of tumour necrosis factor-α converting enzyme (TACE). a) Amino acid sequences for the wild-type TIMP-1 (T1; top); GPI-tethered TIMP-1 (T1Pr; middle); and a GPI-tethered TIMP variant named ‘T1Pr\textsuperscript{α}TACE’ (bottom). Originally tailored for the inhibition of the A disintegrin and metalloproteinases (ADAM) proteinases, T1Pr\textsuperscript{α}TACE has a higher affinity (\(K_i\) app 0.14 nM (standard deviation (SD) 0.06)) for TACE than the wild-type TIMP-1 (356 nM (SD 87)).13,14 b) Membrane localization of GPI-anchored T1Pr and T1Pr\textsuperscript{α}TACE in RAW264.7 cells as shown by non-permeabilized immunofluorescence (IF). Intense membrane staining was detected only in the cells transduced with T1Pr and T1Pr\textsuperscript{α}TACE (blue, 4′,6-diamidino-2-phenylindole (DAPI)-stained nucleus; red, TIMP staining). c) Sequestration of T1Pr and T1Pr\textsuperscript{α}TACE to the cell membrane as shown by reverse zymography and immunoblotting of the membrane extracts and conditioned media. The presence of wild type TIMP-1 in the conditioned media is highlighted by asterisk (*). RZ, reverse zymography; WB, western blot. d) T1Pr\textsuperscript{α}TACE was a potent inhibitor of TACE that reduced TNF-α shedding by > 90%. e) T1Pr\textsuperscript{α}TACE had a negligible effect on the expression of TACE protein. GAPDH, glyceraldehyde 3-phosphate dehydrogenase. Statistical significance between different groups was determined by analysis of variance.
osteoclast differentiation (Figure 4b). Of note, MMP-9 produced by T1Prα-TACE cells remained mostly in latent form (102 kDa), which migrated at a slightly slower pace than matured MMP-9 (92 kDa) in a non-reducing zymography gel (Figure 4b). The findings confirmed that pro-MMP-9 was not able to be processed to the matured form in T1Prα-TACE cells.

Complete abrogation of CatK expression in T1Prα-TACE-transduced cells. Figure 5a summarizes the immunoblot profile of CatK in differently transduced RAW264.7 cells 120 hours post-induction with RANKL. While CatK expression increased dramatically in the control, T1, and T1Prα cells upon induction with RANKL, no CatK could be detected in T1Prα-TACE. Time course analysis showed that CatK protein appeared in the control cells 72 hours following exposure to RANKL, and remained stable until the end of the experiment at 120 hours. In contrast, no CatK could be identified in T1Prα-TACE throughout the entire course of this study (Figure 5b). The findings were supported by IF microscopy results, which revealed a complete absence of CatK in T1Prα-TACE cells that had been RANKL-induced for 120 hours (Figure 5c).

**T1Prα-TACE suppressed the mRNA expression of osteoclast-specific marker genes.** To elucidate the effects of T1Prα-TACE at the genetic level, real-time reverse transcription quantitative polymerase chain reaction (RT-qPCR) was performed on osteoclast-specific marker genes including CatK, TRAP, DC-STAMP, osteoclast stimulatory transmembrane protein (OC-STAMP), ATPase H+-transporting V0 subunit d2 (ATP6V0D2), and osteoclast-associated receptor (OSCAR). Also included in the study was NFATc1, the master transcription factor that regulates terminal osteoclast differentiation. Transcriptional activities of CatK and OC-STAMP were found to be abrogated to pre-induction level in T1Prα-TACE (Figure 6). For TRAP and ATP6V0D2, at least 60% reduction in the mRNA levels was noted in T1Prα-TACE. DC-STAMP and OSCAR were the least affected as only a moderate 25% to 30% downregulation was observed in their mRNA contents. Overall, the data were in agreement with the findings from previous TRAP staining and
immunoblotting studies (Figures 4 and 5). Consistent with the zymography results (Figure 4b), no significant alteration in the mRNA expression of MMP-9 gene was detected in T1PrαTACE. A finding of particular interest was the near-complete abrogation of NFATc1 mRNA in T1PrαTACE. T1PrαTACE prevented NFATc1 from autoregulating its own expression. Next, we probed the protein expression of NFATc1 in osteoclast progenitor cells transduced with different TIMP variants. A > 98% reduction in NFATc1 protein was observed in the cells that had been transduced with T1PrTACE, but not the other TIMPs (Figure 7a). Time course analysis revealed that NFATc1 was more readily degraded in T1PrTACE cells, as evidenced from the multiple lower molecular weight bands on the immunoblots (Figure 7b). The findings were corroborated by IF study, which revealed a marked reduction of NFATc1 in T1PrTACE cells that had been subjected to RANKL induction for 120 hours (Figure 7c).

We next examined nuclear translocation of NFATc1 in the cells that had been induced with RANKL for 24 hours.
NFATc1 was clearly visible within the nuclei of both the control and T1PrαTACE cells, confirming that nuclear translocation of NFATc1 was unaffected by T1PrαTACE at least during the early phase of osteoclast differentiation (Figure 7d).

To gain further insight into how T1PrαTACE interfered with the transcriptional profile of NFATc1, we quantitated the NFATc1 mRNA at 0, 24 hours, 48 hours, 72 hours, and 120 hours following treatment with RANKL. The results agreed with the findings from the immunoblot study, in that NFATc1 mRNA underwent decay more rapidly in the cells that expressed T1PrTACE. Despite an early moderate induction at 24 hours, the level of NFATc1 mRNA in T1PrTACE cells was rapidly reduced to that of the pre-induction level by 120 hours (Figure 7e). Overall, the transcriptional activity of NFATc1 in T1PrTACE was far lower than that of the control cells. The findings confirmed that T1PrTACE prevented NFATc1 from sustaining its own transcription, a process crucial for the lengthy process of osteoclast differentiation.
T1PrαTACE induced sustained activation of p44/42 MAPK to inhibit NFATc1 autoamplification. To examine the effects T1PrαTACE has on RANK protein and MAPK signalling pathways, we next subjected the cell lysates to immuno blotting with p38 and p44/42 antibodies. The results confirmed that, whereas expression of RANK and phosphorylation of p38 MAPK remained relatively unchanged, the level of phosphorylated p44/42 MAPK was more intense and longer-lasting in cells transduced with T1PrαTACE (Figures 8a and 8b). Of note, residual expression of phosphorylated p44/42 MAPK was detectable in T1PrαTACE even after 24 hours of induction.

To investigate if T1 PrαTACE was unique in suppressing osteoclastogenesis, two mutants of different biophysical characteristics were created: 1) T1αTACE, a soluble TIMP mutant with the same sequence as that of T1PrαTACE except the GPI anchor; and 2) T1PrαMT1, a GPI-anchored TIMP mutant designed specifically for the inhibition of MT1-MMP ($K_{i}^{app}$ 1.66 ± 0.17 nM for MT1-MMP$^{20,21}$) (Figure 9a). Reverse zymography and immunoblotting studies confirmed that T1αTACE was highly soluble, whereas T1PrαMT1 was sequestered to the membrane fraction (Supplementary Figure b). Despite sharing similar sequences as T1PrαTACE, T1αTACE and T1PrαMT1 were not able to suppress TRAP expression, thus confirming the uniqueness of T1PrαTACE as a negative regulator of osteoclastogenesis (Figure 9b).

The anti-osteoclastogenic potency of T1PrαTACE was not related to its metalloproteinase-inhibitory activities. To explore if RANKL-mediated osteoclastogenesis required the proteolytic activities of MMPs and TACE, batimastat (a broad spectrum inhibitor active against the MMPs and TACE), ilomastat (a mainly MMP inhibitor), and TAPI-0 (a TACE inhibitor) were added to the cells during the induction process (0.5 to 10 μM). None of the inhibitors were able to prevent osteoclast differentiation irrespective of their concentrations (Figure 9c). The results confirmed that T1PrαTACE attenuated osteoclastogenesis via a
Fig. 6

T1Pr downregulated a broad range of key osteoclast genes. Reverse transcription quantitative polymerase chain reaction analysis revealed a marked downregulation of key osteoclast-specific genes tartrate-resistant acid phosphatase (TRAP), cathepsin K (CatK), osteoclast stimulatory transmembrane protein (OC-STAMP), dendritic cell-specific transmembrane protein (DC-STAMP), ATPase H+ transporting V0 subunit d2 (ATP6V0D2), and osteoclast-associated receptor (OSCAR) in T1Pr cells. A 98% decrease in messenger RNA (mRNA) expression was also revealed in nuclear factor of activated T cells 1 (NFATc1). In contrast, no significant change was detected in the level of matrix metalloproteinase (MMP)-9 mRNA, thus corroborating the findings of the zymography study in Figure 4b. n.s., not significant; RANKL, receptor activator of nuclear factor kappa-B ligand. P-values were calculated using paired t-test.
T1\textsuperscript{Pr\alpha TACE} prevented nuclear factor of activated T cells 1 (NFATc1) autoamplification required for the process of osteoclast differentiation. a) A > 98% downregulation in NFATc1 protein (highlighted by asterisk * ) was detected in RAW264.7 cells transduced with T1\textsuperscript{Pr\alpha TACE} 72 hours after induction with receptor activator of nuclear factor kappa-B ligand (RANKL) (quantification with ImageJ (National Institutes of Health, USA)). b) NFATc1 was more susceptible to degradation in T1\textsuperscript{Pr\alpha TACE} as evidenced from the multiple lower molecular weight bands on the immunoblot (highlighted by asterisk * ). c) Immunofluorescence (IF) staining revealed a lower expression of NFATc1 in T1\textsuperscript{Pr\alpha TACE} cells 72 hours post-induction with RANKL (blue colour: 4',6-diamidino-2-phenylindole (DAPI) staining of the nucleus; red colour: IF staining of NFATc1). d) T1\textsuperscript{Pr\alpha TACE} had no effect on nuclear translocation of NFATc1. Orthogonal views captured with a confocal microscope showing nuclear translocation of NFATc1 in control and T1\textsuperscript{Pr\alpha TACE} cells induced with RANKL for 24 hours (blue colour: DAPI staining of the nucleus; red colour: IF staining of NFATc1). e) T1\textsuperscript{Pr\alpha TACE} prohibited NFATc1 from sustaining its own transcription. Reverse transcription quantitative polymerase chain reaction quantification of NFATc1 messenger RNA (mRNA) at 0 hrs, 24 hrs, 48 hrs, 72 hrs, and 120 hrs after treatment with RANKL. Despite an early induction at 24 hours, the level of NFATc1 mRNA in T1\textsuperscript{Pr\alpha TACE} was rapidly reduced to that of the pre-induction level by 120 hours. Statistical significance was determined by paired t-test.
mechanism unrelated to its metalloproteinase-inhibiting activities.

**Discussion**

Uncontrolled osteoclastogenesis results in increased susceptibility to bone fractures and pathological diseases such as Paget's disease and osteoporosis. Designing novel drugs that target RANKL–RANK interaction and the immediate downstream signalling cascades could potentially revolutionize future pharmacological treatment of many diseases associated with bone loss including osteoporosis, arthritis, and bone cancers. Among the osteoclast-specific gene markers, CatK and TRAP are the most studied due to their critical involvement in bone restructuring.

Our strategy to prevent bone resorption with TIMPs is novel and hitherto unattempted. In this study, we demonstrate that osteoclastogenesis was inhibited at both the transcriptional and translational levels by T1Prα-TACE. The fact that none of the hydroxamate inhibitors were able to prevent osteoclast differentiation indicates that the antosteoclastogenic activity of T1Prα-TACE is unrelated to its metalloproteinase-inhibitory activity. A finding of particular interest was the selectivity of T1Prα-TACE in prolonging p44/42 MAPK activation. NFATc1 production can be induced by activating MAPKs such as p38 and p44/42 (also known as extracellular signal-regulated kinase) through the binding of RANKL to RANK. Interestingly, constitutive activation of p44/42 MAPK has been shown to impede osteoclast differentiation. In the case of Yi et al, a neutralizing antibody to CD9 was able to suppress RANKL-mediated osteoclastogenesis by inducing a stronger and longer p44/42 phosphorylation. This selective activation of MAPK was similar to the effect of T1Prα-TACE we observed in this study. We surmised that the failure of T1Prα-TACE cells in undergoing osteoclast differentiation could be attributed, at least in part, to the effect of TIMP on p44/42 MAPK phosphorylation. We are currently identifying the molecular mechanism

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**Fig. 8**

Selective and sustained activation of p44/42 mitogen-activated protein kinase (MAPK) in T1Prα-TACE cells in response to receptor activator of nuclear factor kappa-B ligand (RANKL) induction. a) T1Prα-TACE had a negligible effect on the protein expression of RANK throughout the process of osteoclastogenesis. b) Selective and sustained activation of p44/42 MAPK in T1Prα-TACE cells. Note the residual expression of phosphorylated p44/42 (highlighted by asterisk *), but not p38 MAPK, in T1Prα-TACE cells even after 24 hours of induction. P-p38 and P-p44/42 denote the phosphorylated forms of p38 and p44/42 MAPKs, respectively.
T1TACE was unique as a negative regulator of osteoclastogenesis. a) Amino acid sequences for T1TACE and T1PrMT1. T1TACE was a soluble tissue inhibitor of metalloproteinases (TIMP) that bore the same amino acid sequence as T1PrMT1 except for the glycosylphosphatidylinositol (GPI) signal peptide. T1PrMT1 was a GPI-anchored TIMP-1 variant designed specifically for the inhibition of MT1-matrix metalloproteinase (MMP). b) Despite sharing similar sequences with T1PrMT1, T1TACE and T1PrMT1 were unable to inhibit osteoclast differentiation. c) None of the hydroxamate inhibitors batimastat (a broad-spectrum inhibitor active against MMPs and tumour necrosis factor-α converting enzyme (TACE)), ilomastat (a mainly MMP inhibitor), and TNF alpha processing inhibitor-0 (TAPI-0) (a TACE inhibitor) were able to inhibit receptor activator of nuclear factor kappa-B ligand (RANKL)-mediated osteoclast differentiation. The data confirmed that the potency of T1TACE was derived from a mechanism independent of its metalloproteinase-inhibitory activity. n.s., not significant.
underlying T1ProTACE/p44/42 MAPK signalling cascade(s) that led to an abrogation in NFATc1 autoamplification.

Based on the findings here, we can conclude that T1ProTACE inhibits RANKL-mediated osteoclast differentiation primarily by preventing NFATc1 autoregulation. Acting possibly by prolonging p44/42 MAPK phosphorylation, T1ProTACE blocked NFATc1 from regulating its own expression. As the key transcriptional regulator pivotal for osteoclast differentiation, NFATc1 upregulates a broad range of osteoclast-specific markers that include TRAP, CatK, DC-STAMP, OC-STAMP, ATP6V0D2, and OSCAR. 

CatK in particular is renowned for its dual abilities in bone resorption and MMP-9 activation.7 As in the case of T1ProTACE, a depletion in NFATc1 leads to an abrogation of these markers and a functionally incapacitated osteoclast progenitor cell that failed to carry out MMP-9 activation or undergo cell fusion in response to RANKL induction.

NFATc1 also mediates MMP-9 transcription.27 Our data showed that MMP-9 transcription was not adversely affected by the downregulation in NFATc1. The failure of T1ProTACE to abrogate MMP-9 transcription suggests that the signalling mechanism that regulates MMP-9 production during osteoclast differentiation is likely to be different from that for TRAP and CatK. TNF-α potentiates osteoclast differentiation by upregulating osteoclastogenic cytokines M-CSF and RANKL in osteoblast-like cells.10 As a potent TACE inhibitor, T1ProTACE is on its own capable of blocking the release of TNF-α to further diminish the occurrence of osteoclastogenesis potentiated via the TNF-α pathway.

In conclusion, we have presented here concrete evidence to demonstrate the efficiency of T1ProTACE as a lead biologic in the suppression of osteoclastogenesis. By incorporating T1ProTACE into a delivery platform such as adeno-associated virus, we hope to develop the TIMP into a medicine suitable for the treatment of diseases characterized by osteoclast hyperactivity. An alternative approach would be to incorporate T1ProTACE into the genome of the target cells using gene editing tools such as clustered regularly interspaced short palindromic repeats (CRISPR) for a more personalized, bespoke treatment.31 In addition to this, we are investigating the mechanism by which T1ProTACE disrupts RANKL-RANK signalling cascades using immunoprecipitation and different omics approaches. The data presented in this study have provided us with new insights into the pathogenesis of osteoclastogenesis and possibly new strategies for antiresorptive drug development.

Supplementary material

Table of polymerase chain reaction (PCR) primers used for quantifying osteoclast marker gene expression by reverse transcription (RT)-qPCR; figures showing results of tartrate-resistant acid phosphatase and phalloidin staining, and reverse zymography and immunoblotting.

References

1. Takito J, Inoue S, Nakamura M. The sealing zone in osteoclasts: A self-organized structure on the bone. Int J Mol Sci 2018;19(4):E964.
2. Chiu YH, Ritchlin CT. DC-STAMP: A key regulator in osteoclast differentiation. J Cell Physiol 2016;231(11):2402–2407.
3. Bi H, Chen X, Gao S, et al. Key triggers of osteoclast-related diseases and available strategies for targetted therapies: A review. Front Med (Lausanne) 2017;4:234.
4. Bossard MJ, Tomaszek TA, Thompson SK, et al. Proteolytic activity of human osteoclast cathepsin K. Expression, purification, activation, and substrate identification. J Biol Chem 1998;271(21):12517–12524.
5. Garnero P, Borel O, Byrjalsen I, et al. The collagenolytic activity of cathepsin K is unique among mammalian proteinases. J Biol Chem 1998;273(48):32347–32352.
6. Kafenah W, Brömme D, Buttle DJ, Croucher LJ, Hollandar AP. Human cathepsin K cleaves native type I and II collagens at the N-terminal end of the triple helix. Biochem J 1998;331( Pt 3):577–732.
7. Christensen J, Shastri VP. Matrix-metalloproteinase-9 is cleaved and activated by cathepsin K. BMC Res Notes. 2015;8:332.
8. Costa AG, Cesano NE, Silva BC, Cremers S, Bilezikian JP. Cathepsin K: its skeletal actions and role as a therapeutic target in osteoporosis. Nat Rev Rheumatol. 2011;7(8):447–456.
9. Lee MH, Murphy G. Matrix metalloproteinases at a glance. J Cell Sci. 2004;117(Pt 18):4015–4016.
10. Lee MH, Rapti M, Murphy G. Unveiling the surface epitopes that render tissue inhibitor of metalloproteinase-1 inactive against membrane type 1-matrix metalloproteinase. J Biol Chem. 2003;278(41):40224–40230.
11. Amour A, Stoccombe PM, Webster A, et al. TNF-α-alpha converting enzyme (TACE) is inhibited by TIMP-3. FEBS Lett. 1998;435(1):30–44.
12. Schon J, Chabla J, Paudel S, et al. Expression profile of matrix metalloproteinases in the labrum of femoroacetabular impingement. Bone Joint Res. 2020;9(4):173–181.
13. Lee M-H, Maskos K, Knüper V, Dodds P, Murphy G. Mapping and characterization of the functional epitopes of tissue inhibitor of metalloproteinases (TIMP)-3 using TIMP-1 as the scaffold: a new frontier in TIMP engineering. Protein Sci. 2002;11(10):2493–2503.
14. Asagiri M, Sato K, Usami T, et al. Autopamification of NFATc1 expression determines its essential role in bone homeostasis. J Exp Med. 2005;202(9):1261–1269.
15. Balkan W, Martinez AF, Fernandez I, Rodriguez MA, Pang M, Troen BR. Identification of NFAT binding sites that mediate stimulation of cathepsin K promoter activity by RANK ligand. Gene. 2005;348(2):90–98.
16. Hawkes SP, Li H, Taniguchi GT. Zymography and reverse zymography for detecting mmps and timp3. Methods Mol Biol. 2010;622:257–269.
17. Zafi D, Neff F, Nagano K, et al. The actin-binding protein coflin and its interaction with coracin are required for podosome patterning in osteoclasts and bone resorption in vivo and in vitro. J Bone Miner Res. 2016;31(9):1701–1712.
18. Toth M, Chyrvkova I, Bernardo MM, Hernandez-Barrantes S, Fridman R. Pro-MMP-9 activation by the MT1-MMP/MMP-2 axis and MMP-3: role of TIMP-2 and plasma membranes. Biochem Bioph Res Commun. 2003;308(2):386–395.
19. Jiang B, Liu J, Lee MH. Targeting A designer TIMP-1 to the cell surface for effective MT1-MMP inhibition: a potential role for the prion protein in renal carcinoma therapy. Molecules. 2019;24(2):E255.
20. Jiang B, Xu Y, Zheng Y, Lee MH. Translocating a high-affinity designer TIMP-1 to the cell membrane for total renal carcinoma inhibition: Putting the prion protein to good use. Mol Cell Biol. 2019;39(18):e00128-19.
21. Dai R, Wu Z, Chu HY, et al. Cathepsin K: the action in and beyond bone. Front Cell Dev Biol. 2020;8:433.
22. Ljusberg J, Wang Y, Läng P, et al. Proteolytic excision of a repressive loop domain in tartrate-resistant acid phosphatase by cathepsin K in osteoclasts. J Biol Chem. 2005;280(31):28370–28381.
23. Park JH, Lee NK, Lee SY. Current understanding of RANK signaling in osteoclast differentiation and maturation. Molecules. 2011;16(10):706–712.
24. Hotokezaka H, Sakai E, Kanazawa K, et al. U0126 and PD98059, specific inhibitors of MEK, accelerate differentiation of RAW264.7 cells into osteoclast-like cells. J Biol Chem. 2002;277(49):47366–47372.
25. Yi T, Kim H-J, Cho J-Y, et al. Tetrapsin CD9 regulates osteoclastogenesis via regulation of p44/42 MAPK activity. Biochem Biophys Res Commun. 2006;347(1):178–184.
Sundaram K, Nishimura R, Senn J, Youssef RF, London SD, Reddy SV. RANK ligand signaling modulates the matrix metalloproteinase-9 gene expression during osteoclast differentiation. Exp Cell Res. 2007;313(1):168–178.

Kim JH, Kim N. Regulation of NFATc1 in osteoclast differentiation. J Bone Metab. 2014;21(4):233–241.

Kim K, Lee SH, Ha Kim J, Choi Y, Kim N. NFATc1 induces osteoclast fusion via up-regulation of Atp6v0d2 and the dendritic cell-specific transmembrane protein (DC-STAMP). Mol Endocrinol. 2008;22(1):176–185.

Asagiri M, Takayanagi H. The molecular understanding of osteoclast differentiation. Bone. 2007;40(2):251–264.

Fitzgerald J. Applications of CRISPR for musculoskeletal research. Bone Joint Res. 2020;9(7):351–359.

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