

Multi-specific targeting of CD44 and the catalytic and hemopexin domains of MMP9 as a therapeutic strategy for osteoclast inhibition

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ARTICLE INFO

Keywords:

CD44 receptor
Hemopexin domain
Metalloproteases
Multi-specific inhibitor
Osteoclast
Tissue inhibitor of matrix metalloproteinase

ABSTRACT

Bone pathologies, such as osteoporosis, are characterized by excessive activity of osteoclasts (OCs)—bone-resorbing cells. As a therapeutic strategy to modulate this activity, we designed a chimera molecule that simultaneously inhibits two key regulators of OC function, matrix metalloproteinase 9 (MMP9) and CD44. The novel fusion molecule, designated C9-PEX, where C9 is a variant of tissue inhibitor of matrix metalloproteinase 2 (N-TIMP2) and PEX is the hemopexin domain in MMP9, exhibits strong binding affinity for CD44 and effectively inhibits the activity of the MMP9 catalytic domain, with an apparent low-picomolar K_i under our fluorogenic assay conditions. In addition, cell-binding assays showed enhanced binding of C9-PEX vs. comparative control proteins, C9 and Ala-C9-PEX. In murine bone marrow-derived OC cultures, gelatin zymography showed that C9-PEX significantly inhibited OC-secreted MMP9, whereas OC differentiation and bone-slice resorption assays showed reduced OC differentiation and reduced OC-mediated bone resorption without detectable toxic effects on cell proliferation and survival. At matched nominal concentrations, C9-PEX consistently outperformed the comparative control proteins and a 1:1 C9 + PEX mixture, supporting a functional advantage of simultaneous target engagement within a single fusion format. Although binding-site/valency-equivalent dosing, formal combination quantification, and normalization of resorption area to OC number on bone slices were not applied in the present study, these findings support C9-PEX as a promising multi-specific inhibitor of OC-mediated resorption in a murine in vitro system. Future studies using human OCs and in vivo models will be required to define the translational efficacy of C9-PEX and the exposure levels required for therapeutic activity.

Introduction

Overactivity of osteoclasts (OCs), bone resorbing cells, is associated with bone pathologies such as periodontitis, rheumatoid arthritis, Paget's disease, tumor-induced osteolytic bone metastasis and osteoporosis [1–6]. In osteoporosis, a chronic bone disease of both women and men over the age of 50, enhanced bone resorption by OCs disrupts the balance between osteoblastic bone formation and osteoclastic bone resorption, leading to the formation of low-density bone that is prone to fractures [6]. With a recent meta-analysis-based estimate of a global prevalence of 18.3% [7], osteoporosis thus constitutes a significant

burden on society and the economy [8]. As a result, intensive R&D efforts are being invested to provide alternative therapies to the commonly used bisphosphonate-based medications [9], which have long retention times that obviate flexible dosing regimens [10] and significant side effects [11,12]. Many of these research efforts target the pathways by which OCs develop and exert their activity. OCs develop from hematopoietic stem cells (HSC), upon activation by the cytokines, macrophage colony stimulating factor (M-CSF) and the receptor activator of NF- κ B (RANK) ligand (RANKL) [13].

Among the newer therapeutic options with superior pharmacokinetic profiles to bisphosphonates, is denosumab, a fully human

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<https://doi.org/10.1016/j.nbt.2026.05.002>

Received 1 March 2026; Received in revised form 3 May 2026; Accepted 4 May 2026

Available online 5 May 2026

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recombinant monoclonal antibody (mAb) that binds to RANKL, preventing it from interacting with its receptor, RANK. Since the RANKL-RANK signaling pathway is essential for OC differentiation and activation, this inhibition reduces bone resorption [14]. Denosumab is not incorporated into bone, making its inhibitory effect reversible and allowing a more flexible dosing regime than bisphosphonates, while its relatively long half-life ensures sustained suppression of bone resorption [15,16]. However, because denosumab targets a single pathway (RANKL-RANK), the body may upregulate compensatory mechanisms, potentially limiting its long-term therapeutic efficacy [17].

Another innovative approach to treating osteoporosis – one that addresses the compensatory upregulation – involves a modified M-CSF, namely, M-CSF with an arginine-glycine-aspartic acid sequence (M-CSF_{RGD}). This modified cytokine binds to and inhibits two key regulators of OC-mediated bone resorption, the cross-interacting receptors $\alpha_v\beta_3$ integrin and c-FMS [18], where $\alpha_v\beta_3$ integrin facilitates the cytoskeletal rearrangement that polarizes the resorptive machinery at the bone–cell interface, and c-FMS regulates OC differentiation and function through its influence on M-CSF signaling. A dual inhibitor of these two functionally interconnected targets is likely to reduce the probability of restoring OC function through compensatory mechanisms. However, the downside of such an approach lies in the necessity for the two membrane proteins, c-FMS and $\alpha_v\beta_3$, to lie in close proximity to one another, which does not always happen. Therefore, for the management of osteoporosis, there is a pressing need for new efficient drugs that can overcome the limitations discussed above.

A potential way to overcome the limitations associated with M-CSF_{RGD} is to select the two targets such that one is anchored to the cell membrane and the other is cell secreted (i.e., soluble). This approach would facilitate effective inhibition without the requirement for the two targets to be in close proximity. Application of this strategy for manipulating OC function could involve dual targeting of a membrane-associated regulator of OC activity, e.g., the cell surface adhesion receptor CD44, and a soluble factor involved in extracellular matrix (ECM) remodeling, such as matrix metalloproteinase 9 (MMP9), which is the MMP most highly expressed by OCs during bone resorption [19]. MMP9 is made up of multiple domains, including a catalytic domain (MMP9_{CAT}) and a hemopexin (PEX) domain [20]. Via its catalytic domain, MMP9 plays a critical role in degrading collagen in the ECM, leading to OC migration, fusion and survival, and bone resorption [19], whereas the PEX domain serves primarily to control substrate specificity [21]. CD44, which is highly expressed by OCs and is equally important for their function, influences multiple intracellular signaling pathways crucial for OC differentiation and activity [22]. Notably, CD44 binds to the PEX domain of MMP9 during OC migration, forming a CD44/MMP9 complex on the cell surface, which promotes activation of pro-MMP9 and contributes to maintaining MMP9 in its functional catalytic form [19,23,24]. Importantly, it has been shown that OCs lacking CD44 fail to produce mature MMP9 [23].

The rationale for utilizing an inhibitor that targets CD44 derives from studies reporting that the binding of CD44 to its natural ligand, hyaluronic acid, led to decreased MMP9 production [24] and that CD44 knockout resulted in downregulation of the NF- κ B/NFATc1 pathway, resulting in decreased OC differentiation and activity [22]. Our working hypothesis for this study was thus that multi-specific inhibition of CD44 and MMP9 could offer a promising therapeutic strategy for OC inhibition in osteoporosis. We posited that such a dual targeting strategy would (i) minimize any potential compensatory activities by CD44 or MMP9, (ii) use the cell-secreted MMP9 as a sink for increasing the local concentration of the bispecific inhibitor near the membrane-anchored CD44, and (iii) enhance avidity and facilitate synergistic inhibition of MMP9 and CD44.

For dual binding to MMP9 and CD44, we utilized a chimera protein previously developed in our laboratory [25] that was judged to be a particularly promising inhibitor of OC activity. This chimera, designated C9-PEX, comprises a variant of the N-terminal domain of tissue inhibitor

of matrix metalloproteinase 2 (N-TIMP2), designated C9, fused to the PEX domain of MMP9. Herein, we show that the dual binding affinity of the chimera for MMP9 and CD44 derives from the strong affinity of C9 for MMP9_{CAT} and the ability of the PEX domain to bind to CD44. We show that C9-PEX exhibits strong binding affinity for CD44 and inhibits MMP9_{CAT} with an apparent low-picomolar K_i under our fluorogenic assay conditions, and that – in comparison to its comparative control proteins (see below) – C9-PEX shows stronger inhibitory activity of MMP9 secreted from OCs, of OC formation and of OC-mediated bone resorption, without exerting any toxic effects on cell proliferation and survival.

Material and Methods

Ethics statement

This study was carried out according to relevant guidelines and regulations and the protocols were approved by the Ben-Gurion University Committee for the Ethical Care and Use of Animals in Experiments (permit number: IL-67-09-2019 B). This study is reported in accordance with ARRIVE guidelines (<https://arriveguidelines.org>). Mice were euthanized using isoflurane followed by cervical dislocation.

Production and purification of C9 and C9-containing proteins

In previous work in our laboratory, we prepared the multi-specific protein adduct, C9-PEX, and two ‘control’ compounds for purposes of comparison, C9 and A-C9-PEX (which has an alanine residue appended to the N-terminus of C9-PEX), that predominantly bind MMP9 and CD44, respectively, within functional concentration ranges [25]. The genes of the three different C9 proteins were cloned into the pPIC3 α A vector for expression in *Pichia pastoris* strain X33. These C9 proteins were prepared with C-terminal 6 $\times$ His tags, which enabled us to purify them using a nickel-NTA affinity column (Cytiva, USA). Since C9-PEX can form homodimers through the PEX domain, the purification included a gel filtration step on a Superdex 75 column (GE Healthcare Life Sciences, USA) and an ÄKTA pure instrument (GE Healthcare Life Sciences) to separate the monomers from the dimeric form of C9-PEX and to eliminate other impurities (Fig. S1). Protein concentrations were determined by UV-Vis spectroscopy, with absorbance at 280 nm, using a NanoDrop spectrophotometer (ϵ_{280} of 52870 M⁻¹ cm⁻¹ for C9-PEX and A-C9-PEX, 39545 M⁻¹ cm⁻¹ for PEX, and 13325 M⁻¹ cm⁻¹ for C9). The production yield was ~30 mg protein per 1 L of yeast culture, and the correct size of the proteins was confirmed by SDS-PAGE and mass spectrometry analysis (Fig. S1) (Ilse Katz Institute for Nano-scale Science and Technology, BGU, Israel).

The C9 proteins were labeled for 1 h at room temperature (RT) with a DyLight 488 amine reactive dye (ThermoFisher, USA) in 0.05 M sodium borate buffer, pH 8.5, at a molar ratio of 1:10 (protein:dye). The labeled proteins were washed free of unbound dye on a desalting column (Cytiva, USA), connected to ÄKTA Start (GE Healthcare Life Sciences, USA). The purity of the labeled proteins was verified by gel electrophoresis, followed by fluorescence imaging with a Typhoon FLA9500 gel imager (GE Healthcare Life Sciences).

MMP9_{CAT} inhibition studies

The kinetics of the reaction between MMP9_{CAT} (0.325 nM) and its fluorogenic substrate, Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH₂-TFA (Merck Millipore, USA; 7.5 μ M), in the presence of different concentrations of wild-type N-TIMP2 (N-TIMP2_{WT}; control) and the different C9 proteins (0.03125–0.75 nM) were determined using a Synergy 2 plate reader (BioTek, USA). Calculations to obtain K_i values were performed by utilizing Morrison’s equation, the classic competitive inhibition equation for tight binding.

$$\frac{V_i}{V_0} = 1 - \frac{([E] - [I] + K_i^{app}) - \sqrt{([E] - [I] + K_i^{app})^2 - 4[E][I]}}{2[E]} \quad (1)$$

where V_i - enzyme velocity in the presence of inhibitor, V_0 - enzyme velocity in the absence of inhibitor, E - enzyme concentration, I - inhibitor concentration, and K_i^{app} - the apparent inhibition constant, which is given by:

$$K_i^{app} = K_i \left(1 + \frac{[S]}{K_m}\right) \quad (2)$$

where K_i - inhibition constant, and K_m - Michaelis-Menten constant, which was determined elsewhere [26]. Mean K_i values $\pm$ standard error (SEM) were obtained from three technical repetitions.

Surface plasmon resonance (SPR) experiments

Binding of C9-PEX and C9 to MMP9_{CAT} was evaluated using a ProteOn™ XPR 36 instrument (Bio-Rad, USA) to accurately determine the dissociation constants (K_D) of the complexes. C9-PEX and C9 were immobilized on an HC200M chip (XanTec bioanalytics GmbH, Dueseldorf, Germany) using standard amino coupling procedures, namely, activation of the carboxymethyl groups on the chip surface with 20 mM EDAC and 5 mM S-NHS at a flow rate of 30 μ L/min for 300 s. Thereafter, to attach C9-PEX and C9 covalently to the chip, 2.4 μ g of C9-PEX or 3 μ g of C9 were suspended in acetate buffer at pH 5.5 and pH 5, respectively, and allowed to flow over the chip at a flow rate of 30 μ L/s for 300 s. The negative control comprised 5 μ g proline-proline-glutamate protein (PPE), which was bound on another channel and provided a background signal. Unbound carboxyl residues were then deactivated with 1 M ethanolamine at a flow rate of 30 μ L/min for 300 s. MMP9_{CAT} at five different concentrations (i.e., 274, 137, 68.5, 34.25, 17.125 and 0 μ M, with 0 μ M providing a background signal) was allowed to flow over the C9 and C9-PEX immobilized channels at a flow rate of 30 μ L/min for 818 s, followed by a dissociation step of 300 s. The system was operated at 25 °C in PBST (PBS $\times$ 1 with 0.005% Tween 20) as the running buffer. Measurements were acquired using ProteOn Manager 3.1.0, evaluation software (Bio-Rad), from which background signals were subtracted. Affinity was calculated by averaging the response units at equilibrium and plotting this value against each MMP9_{CAT} concentration. Graphs of response units vs. MMP9_{CAT} concentrations were then fitted to the Langmuir 1:1 equilibrium model, and K_D values were calculated.

Micro-scale thermophoresis (MST) affinity measurements

The binding of the C9 proteins to CD44 was determined by MST and analyzed by Monolith NT.115 (NanoTemper Technologies, Germany). C9-PEX, A-C9-PEX and C9 were labeled with DyLight 488, as described above, and the labeled proteins (i.e., ligands at 150 nM) were incubated overnight at a 1:1 ratio with 16 serial dilutions of the analyte, CD44 (Sino Biological, China), at a starting concentration of 5.65 μ M. The mixtures of the C9 proteins with CD44 were then transferred to glass capillaries, and fluorescence intensity was determined in the middle of each capillary (with a 530 nm filter) and plotted against the concentration of the ligand. Steady-state affinity was calculated using NT Control 2.0.2.29 software (NanoTemper Technologies) by measuring the changes in fluorescence (before heating and a few seconds after, i.e., the effect of heat on the fluorescence of the labeled molecule, designated the "temperature jump") against the analyte concentrations. Two baselines were defined: one at a high CD44 concentration, at which most of the C9 protein molecules were bound to CD44, and the other at a low CD44 concentration, at which CD44 was not bound to the C9 molecules. K_D was defined as the concentration at which 50% of the labeled C9 protein was complexed to CD44. To verify that the differences in fluorescence were the result of a specific interaction of CD44 with the ligand, each mixture was subjected to denaturation at 95 °C for 10 min and then

transferred to capillaries, followed by remeasurement of the fluorescence. The difference in the fluorescence values, namely, between the native protein complex and the denatured protein complex, thus represented the net effect on the fluorescence attributable to a specific interaction between the relevant C9 protein and CD44.

Cell-binding assay

Affinity measurements of C9-PEX, A-C9-PEX and C9 – all conjugated to DyLight 488 – for wild-type (WT) MCF-7 cells (ATCC HTB-22) and for an MMP9-positive MCF-7 cell line were then performed. The MMP9-positive MCF-7 cell line had previously been generated in our laboratory by transfecting MCF-7 cells with a pCMV MMP9 plasmid (Sino Biological) using Lipofectamine 3000 (ThermoFisher), according to the manufacturer's instructions [25]. Cells of both lines were grown to 70–80% confluence and then detached from the flasks by using trypsin. For evaluating the binding of PEX to the WT MCF-7 cells, 10⁵ cells were collected for each treatment group in eppendorf tubes and then washed three times with 1% PBSA (1% BSA in PBS), with each washing step being followed by centrifugation at 500 g for 5 min. For determining whether C9-PEX binds to MCF-7 cells in an MMP9-dependent manner, MCF-7 cells (both WT MCF-7 and the MMP9-positive MCF-7 cell line) were incubated with either C9-PEX or A-C9-PEX at two different concentrations (500 nM and 1000 nM) for 1 h at RT. For evaluating the binding of the C9 proteins to cells, WT MCF-7 cells were incubated with DyLight 488-conjugated C9-PEX, A-C9-PEX or C9 at concentrations of 0, 25, 50, 100, 250, 500, 750, 1000, 1200, 1500, 1750 and 2000 nM. Unless otherwise specified, all C9 proteins were used at nominal protein molarity in all the comparative cell-based assays. Cells were then washed three times with 1% PBSA, with each washing step being followed by centrifugation at 500 g for 5 min, and then resuspended in 1% PBSA with DAPI (0.1 μ g/mL) to identify living cells. The cells were then analyzed by FACS (BD FACSCanto™ II flow cytometer, BD Biosciences, USA). As a negative control, cells were analyzed in the absence of added proteins. To verify the expression of CD44, anti CD44 antibody conjugated to allophycocyanin (APC) (Bio-Rad, USA) was added to the cells at dilution of 1:10. The flow cytometry histograms were analyzed using FlowJo vX.0.7.

Inhibition of MMP9 derived from OCs

For this experiment and all subsequent experiments with murine bone marrow derived monocytes (BMMs), the cells were obtained by flushing the bone marrow from the femurs and tibias of 8- to 20-week-old, 25 g WT C57BL/6JRccHsd mice (Harlan, Israel). The cells were treated with ammonium-chloride-potassium (ACK) lysis buffer (ThermoFisher Scientific, USA) and resuspended in a mixture of α -MEM growth medium (Sigma, USA), 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, 1% L-glutamine and 30 ng/mL of recombinant murine M-CSF (R&D systems, USA). The cells were incubated for three days at 37 °C under 5% CO₂ on a sterile uncoated plastic culture plate to select and enrich for monocytes. The cells were then washed with cell medium and detached using a cell scraper, and 2 $\times$ 10⁴ cells were transferred to each well of a 96-well plate supplemented with 30 ng/mL of recombinant murine M-CSF and 50 ng/mL of RANKL (R&D Systems, USA). The medium was refreshed by changing half of the volume every 48 h until OC formation. After OC formation, the OC cell medium (containing secreted MMP9) was loaded onto gelatin-containing gels for analysis by SDS-PAGE. The gels were washed with a buffer containing 4% Triton X-100 in doubly distilled water (DDW) and incubated with 20, 50 and 100 nM of C9, C9-PEX, A-C9-PEX or C9 + PEX (a 1:1 molar ratio mixture of individual C9 and PEX proteins) in development buffer (50 nM Tris pH 7.4, 10 mM CaCl₂, 0.02% NaN₃ in DDW). Gelatine degradation by OC-secreted MMP9 and inhibition of its degradation by the proteins were visualized as white bands (at different levels of intensity) on a dark background after staining with SimplyBlue™

SafeStain (ThermoFisher) and imaging the gels using a gel imager (Vilber, France). The intensity of the white bands was quantified using FIJI 1.54 f software.

CD44 and C9-PEX co-localization assay

OCs were allowed to differentiate in culture, as described above, until mature OCs were observed. Then, the cell culture, containing differentiating CD14 + monocytes and mature OCs, was washed gently three times with 0.1% PBSA (0.1% BSA in PBS) and incubated with 1000 nM C9-PEX for 40 min at 37 °C. Cells were then washed three times with 0.1% PBSA, fixed with 4% formaldehyde for 15 min, and permeabilized using 0.1% Triton X-100 for 10 min, all at RT. Thereafter, cells were washed three times with 0.1% PBSA and incubated with an anti-C-myc antibody conjugated to fluorescein isothiocyanate (FITC) for 1 h at RT. Following the incubation, the cells were washed three times with 0.1% PBSA and incubated at RT with a rat anti-CD44 antibody for 1 h and then with anti-rat Alexa Fluor 568 antibody. Cells were washed three times with 0.1% PBSA, and Hoechst solution was added for staining of the nuclei 10 min before microscope image analysis. Cells were imaged using Cell Discoverer 7 (Zeiss, Germany) at $\times 20$ magnification, and stitched images were obtained using FIJI 1.54 f software.

OC differentiation assay

Murine BMMs were seeded on a 96-well plate as described above. Then, 250, 500 and 1000 nM of C9-PEX, A-C9-PEX, C9 and C9 + PEX were added to the wells in triplicate. Positive control groups were untreated, and negative control groups contained no C9 proteins or no RANKL. The medium was refreshed every 48 h by changing half of the volume in each well with fresh medium containing the C9 proteins until intact OCs were observed. OCs were fixed using 4% formaldehyde for 15 min at RT and stained using a tartrate-resistant acid phosphatase (TRAP) staining kit (Sigma, USA). DAPI solution was added to the cells for staining of the nuclei 10 min before microscope imaging. Each well was imaged using APEXVIEW APX100 benchtop fluorescence microscope (Evident Olympus, Japan) at $\times 20$ magnification, except for the C9 + PEX group and its positive control, which were imaged using Cell Discoverer 7 (Zeiss, Germany). Images from each well were stitched to cover the analyzed well area and then analyzed manually using FIJI 1.54 f software for the evaluation of number of OCs per well. Nuclei were detected and counted by QuPath 0.5.1 software using the StarDist deep learning-based segmentation algorithm [27] with a pretrained model for fluorescently labeled nuclei at full image resolution (1 pixel = $0.5685 \times 0.5685 \mu\text{m}$), and no nucleus expansion was applied. The normalized number of nuclei was calculated as the total number of nuclei inside the OCs divided by the total number of nuclei in a well. In this assay, OCs were defined as TRAP-positive cells with at least three nuclei. Statistical analysis was performed on well-level values, with $n = 3$ replicate wells per treatment condition. Individual OCs, nuclei, and stitched image fields were not treated as independent statistical replicates.

Cell viability test

Murine BMMs (7000) were transferred to each well of a 96-well plate supplemented with 30 ng/mL of recombinant murine M-CSF and 50 ng/mL of RANKL in the presence of different concentrations of C9 proteins (250 nM, 500 nM, and 1000 nM). After 48 h, XTT reagent (Biological Industries, Israel) was added to the cells according to the manufacturer's protocol for 2 h at 37 °C. The plates were rocked gently, and the absorbance signals were measured at wavelengths of 450 nm and 650 nm, with the latter serving as the background absorbance, using a Synergy 2 plate reader (BioTek, USA). The blank absorbance (medium without cells) and the background absorbance were subtracted from the absorbance of each sample (cells plus relevant C9 protein).

OC bone resorption assay

Murine BMMs (2×10^5) were transferred to each well of a 96-well plate containing 30 ng/mL of recombinant murine M-CSF and 50 ng/mL of RANKL. Each well also contained a bone slice (Boneslices.com, Denmark) that had been washed three times with PBS before being placed in the well. C9-PEX, A-C9-PEX, C9, and C9 + PEX were added at final concentrations of 250, 500 and 1000 nM. Positive (untreated) and negative (lacking either C9 proteins or RANKL) control groups were also included. The culture medium was refreshed every 48 h by replacing half of the volume in each well with fresh medium containing the relevant C9 protein. The experiment was run for 14 days, at the end of which the cells were gently scraped off the bone slices by using cotton swabs. Thereafter, the bone slices were rinsed in DDW, and dried completely with paper. Care was taken to maintain the correct orientation of the slices and to keep track of the side on which the cells had grown. To ensure that the resorption pits were properly visualized, each bone slice was placed carefully on a 20- μL droplet of toluidine blue solution (1% toluidine blue, 1% sodium borate in DDW) with the side on which the cells had grown facing downward in direct contact with the stain. The bone slices were then incubated for 4 min at RT and rinsed in DDW to remove excess dye. The stained bone slices were placed in an ibidi 8-well chamber (ibidi, Germany) and imaged at $\times 20$ magnification using an Olympus IX83 microscope with an automated stage (Olympus, Japan). Images from each bone slice were stitched to cover the analyzed bone-slice area and analyzed individually using FIJI 1.54 f software to quantify the total resorption area per bone slice. The resorption area of each well was divided by the total bone slice area and normalized to the untreated group. Statistical analysis was performed on bone slice-level values, with $n = 3$ replicate bone slices per treatment condition. Individual stitched image fields or resorption pits were not treated as independent statistical replicates.

Statistical analysis

The data was analyzed with GraphPad Prism version 8.0.1.244 for Windows (La Jolla, CA, USA). Data is shown as means $\pm$ SEM or SD as indicated in the figure legends. Statistical significance was determined by one-way ANOVA followed by Dunnett's multiple-comparisons test or Student's *t*-test, as indicated in the figure legends. *P*-value < 0.05 was considered statistically significant. All experiments, except for SPR and MST, were performed in triplicate.

Results and discussion

C9-PEX inhibits MMP9CAT

The extent of MMP9_{CAT} inhibition by the different TIMP2-derived inhibitors followed the order C9 > C9-PEX > N-TIMP2_{WT}, with K_i values of 6.82 ± 0.38 pM, 17.29 ± 1.31 pM, and 51.08 ± 3.01 pM, respectively (Fig. 1). Notably, C9-PEX maintained strong inhibition of MMP9_{CAT} activity despite the fusion of the C9 inhibitor of MMP9_{CAT} to the PEX domain.

Fusion to PEX does not disrupt the ability of the C9 inhibitor to bind to MMP9CAT

We next aimed to determine whether the C9-PEX chimera has the same MMP9 binding effect as C9, and if so, then why. For affinity measurements, C9-PEX and C9 were immobilized on an SPR chip, and MMP9_{CAT} as the analyte was allowed to flow over the immobilized proteins. The affinity constants (K_D) so obtained for the complexes were 102 ± 30 nM and 101 ± 36 nM for C9 and C9-PEX, respectively (Fig. 2), suggesting that the PEX domain in C9-PEX does not interfere with the binding of C9 to MMP9_{CAT}. The K_D values were calculated from the binding equilibrium (Fig. 2 C), because the extremely slow dissociation rate both for C9 and C9-PEX from MMP9_{CAT}, indicating on long-lasting

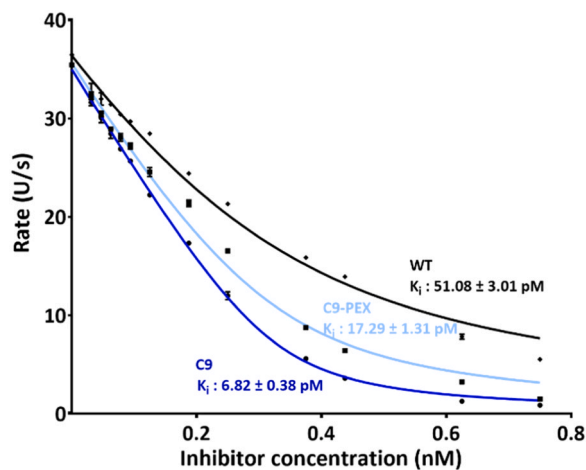


Fig. 1. Substrate degradation rate at different concentrations of the MMP9_{CAT} inhibitors N-TIMP2_{WT}, C9-PEX and C9. The fluorescence signal upon substrate cleavage by MMP9_{CAT} was determined at the different inhibitor concentrations. Thereafter, the cleavage rates at each concentration of inhibitor were determined, and the curves were fitted (using GraphPad Prism 8.0.1) to the Morrison tight binding equation (Eq. 1) to obtain the K_i values ($n = 3$). Error bars represent SEM; error bars smaller than the symbol are not shown. K_i values for each variant are given on the figure.

complex stability, prevented direct calculation of K_D values from the k_{on} and k_{off} rate constants. Nonetheless, the findings do show slow dissociation and strong inhibition, which may indicate the promising therapeutic potential of C9-PEX by ensuring prolonged suppression of MMP9 activity in OCs. In a previous study, we found similar K_D values for a different dual-specific inhibitor, namely M-CSF_{RGD}, which targeted both

c-FMS and $\alpha_v\beta_3$ integrin and subsequently inhibited the OC differentiation that is dependent on their activation [18].

The apparent contradiction between the low-picomolar K_i values obtained in the fluorogenic peptide assay and the nanomolar K_D values obtained by SPR may be attributed to the different properties measured in the two assays—inhibition of MMP9_{CAT} under catalytic conditions vs. equilibrium binding of MMP9_{CAT} to C9 or C9-PEX under non-catalytic conditions. Nonetheless, the values of both K_i and K_D are consistent with tight-binding behavior under catalytic conditions and a slow-dissociating and potentially slow-binding interaction of MMP9 to C9 and C9-PEX. Since the above data does not enable us to posit a full inhibitory mechanism or directly compare the activity-derived K_i with the SPR-derived K_D , the K_i values are referred to as apparent K_i values under the fluorogenic assay conditions.

The PEX domain binds CD44 in vitro

The findings of the MST experiment to examine the binding of the PEX domain to CD44 showed that both C9-PEX and A-C9-PEX (as control proteins for purposes of comparison) bound to CD44, with K_D values of 77.01 ± 25.47 nM and 47.24 ± 10.04 nM, respectively (Fig. 3A, B). As expected, C9 did not bind to CD44 (Fig. 3C). Importantly, the affinities of C9-PEX for MMP9 and CD44 were similar, and since both C9 and PEX domains were present in our experimental system at similar concentrations (in the C9-PEX format), an optimal inhibitory effect should be obtained for both domains by using C9-PEX. Adding a new functionality in the form of the PEX domain to C9, without interfering with the ability of C9 to bind and inhibit MMP9, further indicates the utility of the C9-PEX format, which exhibits stability and the capability of engaging simultaneously with multiple targets. A similar strategy for dual interaction with two targets for inhibiting OC differentiation was previously observed for M-CSF_{RGD} targeting of c-FMS and $\alpha_v\beta_3$ integrin, but the

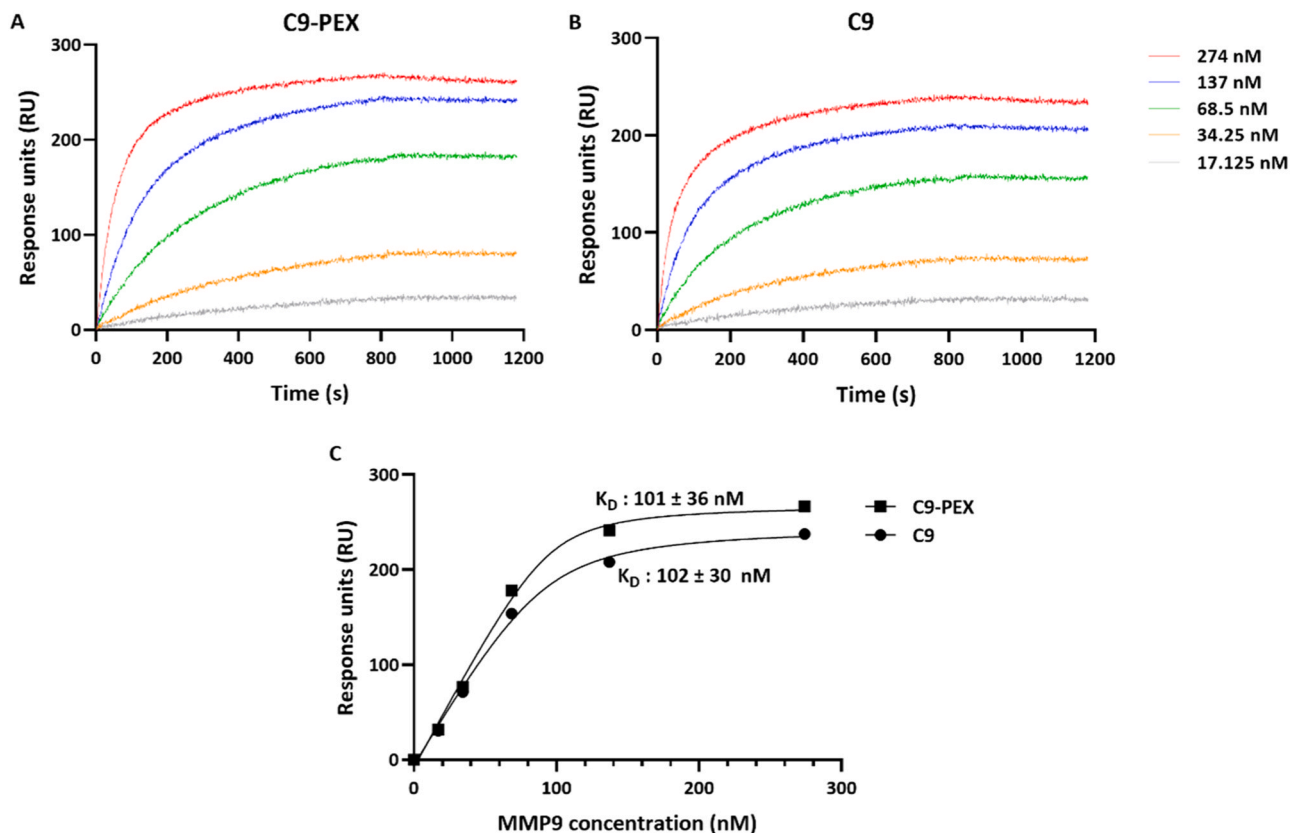


Fig. 2. (A, B) SPR binding of MMP9_{CAT} to the immobilized ligands, C9-PEX and C9, respectively. (C) Equilibration binding analysis for C9 and C9-PEX to obtain K_D values. Graphs were plotted using GraphPad Prism 8.0.1. The K_D value for each variant is presented on the graph.

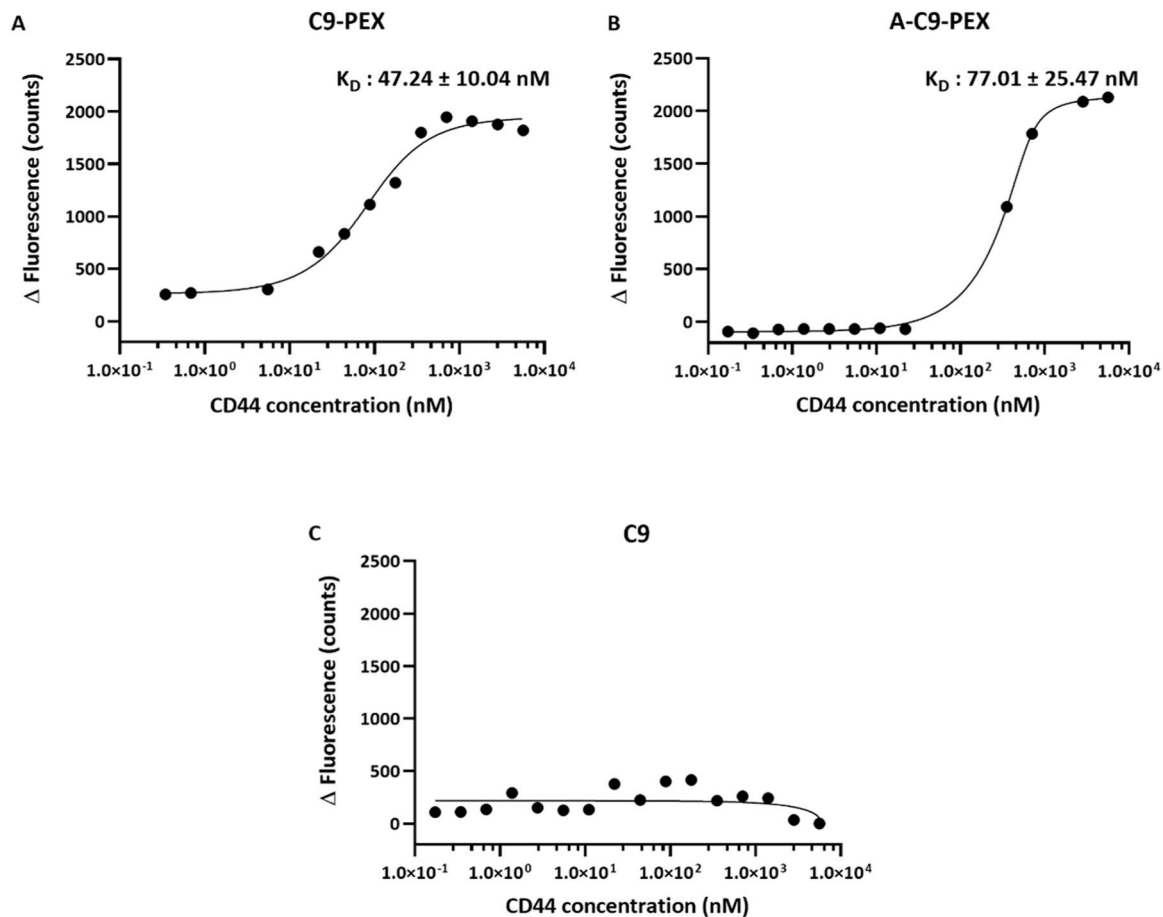


Fig. 3. Binding of the PEX domain to CD44, as determined by MST: Δ fluorescence vs. CD44 concentration for (A) C9-PEX, (B) A-C9-PEX and (C) C9. Samples were analyzed using Monolith NT.115 (NanoTemper Technologies). K_D values were calculated from the fitted curves using the NT Control 2.0.2.29 software (NanoTemper Technologies) and presented using GraphPad Prism 8.0.1. The K_D value for each variant (except for C9) is presented on the graphs.

RGD-containing domain of M-CSF_{RGD}, which has the ability to bind all integrin family members [28], may impair the binding specificity of the modified cytokine.

Binding of C9 proteins to cells via PEX

Affinity titration experiments, designed to evaluate the binding of C9, C9-PEX and A-C9-PEX to cells – in this case WT MCF-7 cells – vs. in solution, showed that in cells CD44 makes a larger contribution than MMP9 to the binding of C9-PEX (despite the similar affinities of C9-PEX to purified soluble MMP9 and CD44). This finding may be attributed to the fact that while CD44 is expressed on the cell membrane [24], MMP9 is largely secreted from the cell [23,24], enabling a direct connection to be formed between C9-PEX and the cells. Nevertheless, it is obvious that the interaction of C9-PEX with the MCF-7 cells was dependent on both CD44 and MMP9, as a stronger binding signal was observed for C9-PEX than for the control proteins, C9 and A-C9-PEX, possibly due to MMP9 serving as a sink, increasing the local concentration of the bispecific inhibitor and hence contributing to enhanced binding to the membrane target, i.e., CD44. Moreover, C9-PEX – but not C9 or A-C9-PEX – exhibited two-phase cell-binding behavior, probably as a result of its interactions with both targets (Fig. 4). Of note, the Ala component of the A-C9-PEX control did not completely abolish binding to MMP9, but residual MMP9 binding was observed only at concentrations above 1500 nM (Fig. 4), which is above the concentration range used in the subsequent functional assays.

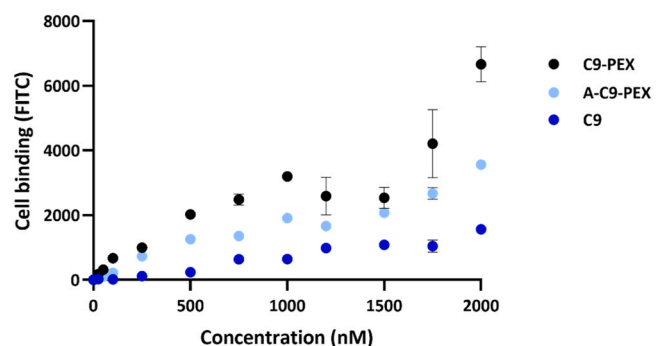


Fig. 4. Flow cytometry analysis showing binding titration curves for C9-PEX, A-C9-PEX and C9 in WT MCF-7 cells. Each experiment was performed with 0, 25, 50, 100, 250, 500, 750, 1000, 1200, 1500, 1750, 2000 nM of the C9 proteins ($n = 3$). Graph was plotted using GraphPad Prism 8.0.1. Error bars represent SEM. Error bars smaller than the symbol are not shown.

C9-PEX binds to MCF-7 cells in an MMP9-dependent manner

The results obtained from the above cell-binding assay confirmed the higher contribution of the cell-membrane-expressed receptor CD44 vs. the secreted MMP9 for C9-PEX binding, but we nevertheless sought to isolate the contribution of C9 to binding of C9-PEX to cells, i.e., the C9-PEX binding dependency on MMP9 secretion from the cells. For that purpose, we compared the affinities of C9-PEX and A-C9-PEX (which lacks MMP9 binding capability) to WT MCF-7 (which secrete only minimal amounts of MMP9) [29] and to MMP9-positive MCF-7 cells

(which overexpress MMP9) [25]. Although both chimera proteins bound to both WT and MMP9-positive MCF-7 cells, the binding superiority of C9-PEX over A-C9-PEX after 1 h of incubation at RT was significantly more pronounced for the MMP9-positive MCF-7 cells than for the WT MCF-7 cells (Fig. 5 and Fig. S2), confirming the contribution of MMP9 to C9-PEX binding to the cells. The higher affinity of C9-PEX (vs. A-C9-PEX) to cells expressing both CD44 (Fig. S3) and MMP9 was therefore a result of its interaction with both targets, probably at the same time. This dual interaction may be facilitated by the interaction of MMP9 with CD44 on the cell membrane (via the PEX domain of MMP9) [19,23,24]. Together with secreted MMP9, which – through binding to C9-PEX – enhances the local concentration of C9-PEX near the cell, colocalization of the membrane-bound MMP9 with CD44 resulted in the superior affinity of C9-PEX (vs. A-C9-PEX) to the cells.

C9-PEX strongly inhibits MMP9 secreted from OCs

A zymography assay used to evaluate the ability of C9-PEX to inhibit MMP9 secreted from OCs showed, as expected, that both C9-PEX and C9, but not A-C9-PEX, exhibited significant inhibitory activity against MMP9 (Fig. 6). Notably, C9-PEX was more potent than C9 in this assay despite C9 being more potent in inhibiting purified MMP9_{CAT} (Fig. 1), probably due to the contribution of the PEX domain in C9-PEX, which interacts with the PEX domain (via dimerization) of the OC-secreted full-length MMP9 used in this assay [25]. Importantly, at matched nominal concentrations (of either of 50 nM or 100 nM), C9-PEX demonstrated superior inhibition of OC-secreted MMP9 vs. a 1:1 mixture of C9 and PEX (Fig. 6), thereby suggesting that fusion of C9 to PEX to create a single molecule provides a functional advantage over two single molecules in admixture. This enhanced inhibition may stem from the binding of C9-PEX via its PEX domain to the PEX domain of MMP9 via self-association [25,30].

A different approach to MMP9 inhibition relied on the MMP9-specific inhibitor, MMP-9-IN-1, a small molecule inhibitor of MMP9 [31]. That compound demonstrated significant inhibition of the activity of MMP9 secreted by osteoblasts stimulated by oxidative stress, with an approximate 10% reduction in MMP9 activity at an inhibitor concentration of 100 nM. At the same inhibitor concentration (100 nM), C9-PEX exhibited a substantially greater effect, reducing MMP9 activity by about 98% compared to the control group.

C9-PEX binds OC precursors rather than OCs

When C9-PEX was incubated with differentiating CD14 + monocytes, the primary precursors of OCs [32], it was found that C9-PEX

bound to a subset of OC precursors, rather than to mature OCs, which were also present in the cell culture (Fig. 7 and Fig. S5). The mononuclear cells bound to C9-PEX were confirmed to be CD14 + monocytes (Fig. S5). In those cells, C9-PEX appeared to co-localize with CD44 on the cell surface (Fig. 7), suggesting that it may inhibit their further differentiation into OCs, although the binding mechanism is not immediately apparent and requires further investigation.

C9-PEX inhibits OC formation

An examination of the inhibitory effect of C9, C9-PEX, A-C9-PEX and C9 + PEX on OC formation in cultures of differentiating BMMs revealed that C9-PEX alone significantly inhibited OC formation compared to the untreated control group at a concentration of 500 nM (Fig. 8A, B). Only at a concentration of 1000 nM did both C9-PEX and A-C9-PEX, but not C9, significantly inhibit OC formation (Fig. 8A, B). This outcome is in agreement with our observation in WT MCF-7 cells (Fig. 4) that CD44 had a greater influence than MMP9 on the binding of C9-PEX to OC and hence on the inhibition of OC formation. The finding that C9-PEX is a more potent inhibitor than A-C9-PEX of OC formation (although both have a PEX domain) suggests that the binding of C9-PEX to OCs is promoted by the C9 domain, via its interaction with MMP9 and probably also via improved interaction with the CD44 colocalized with MMP9 on the OC membrane. The superior effect of CD44 vs. MMP9 on the inhibition of OC formation by C9-PEX was also demonstrated in the normalized number of nuclei, where at 1000 nM, C9-PEX, A-C9-PEX and C9 + PEX, but not C9, caused significant decreases in the number of nuclei compared to the untreated control group. A-C9-PEX was expected to exhibit a similar inhibitory profile to C9 + PEX, since both A-C9-PEX and PEX can bind CD44, but the results showed that A-C9-PEX (and also C9-PEX) was more potent than C9 + PEX, possibly due to the stabilization of the PEX domain in A-C9-PEX, thereby facilitating better binding to CD44 and inhibition of OC formation. To rule out nonspecific toxic effects of the different inhibitors on cell proliferation and survival, we investigated the survival and proliferation capabilities of murine BMMs exposed to the C9 proteins. No changes were observed at the concentrations used in the experiment (Fig. S6).

C9-PEX reduces osteoclast-mediated bone resorption

To evaluate the effect of C9-PEX on OC-mediated bone resorption, we conducted a bone resorption assay. This experiment was conducted to investigate the possibility that, despite significant inhibition of OC formation by the C9 proteins, a mechanism compensating for the reduction in OC formation (Fig. 8) might be activated in the remaining

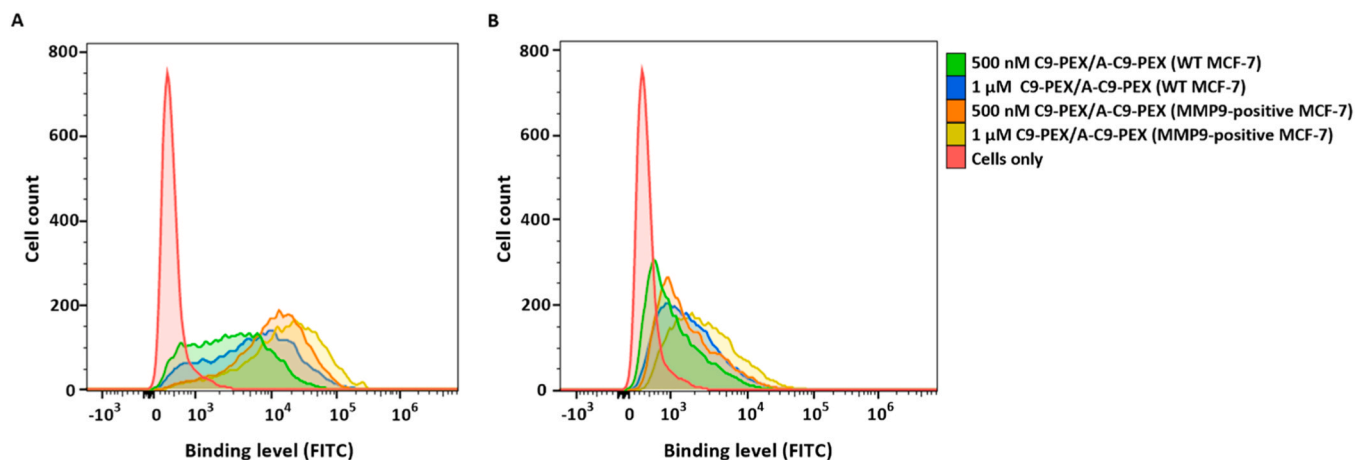


Fig. 5. FITC intensity histograms of (A) C9-PEX and (B) A-C9-PEX in cultures of WT MCF-7 and MMP9-positive MCF-7 cells, showing binding of the proteins to the cells. C9-PEX and A-C9-PEX were conjugated to DyLight 488 and tested for binding to WT MCF-7 and MMP9-positive MCF-7 cells by using flow cytometry. The histograms were analyzed using FlowJo vX.0.7.

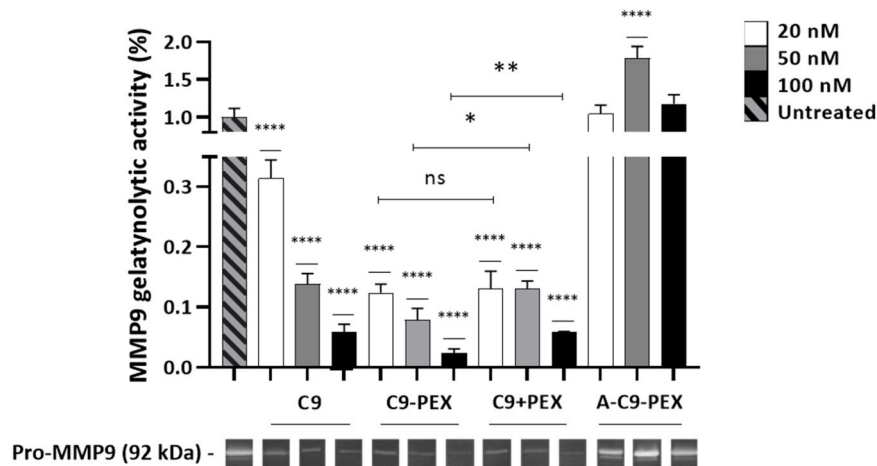


Fig. 6. Gelatin zymography showing the ability of C9 proteins to inhibit MMP9 secreted from OCs. An OC culture medium containing MMP9 was loaded onto a gelatin gel and incubated overnight with the different C9 proteins. One-way ANOVA with Dunnett's multiple comparison to the untreated control was utilized for statistical analysis. Comparison between the C9-PEX group and the C9 +PEX group was tested using Student's *t*-test. * $P \leq 0.05$, ** $P \leq 0.01$ **** $P < 0.0001$, $n = 3$. Error bars represent SD. Graph was plotted using GraphPad Prism 8.0.1. The white bands indicate cleavage of the gelatin by MMP9 (92 kDa). Representative images of the protein bands on the gel are shown under the graph. Original gels are presented in Fig. S4.

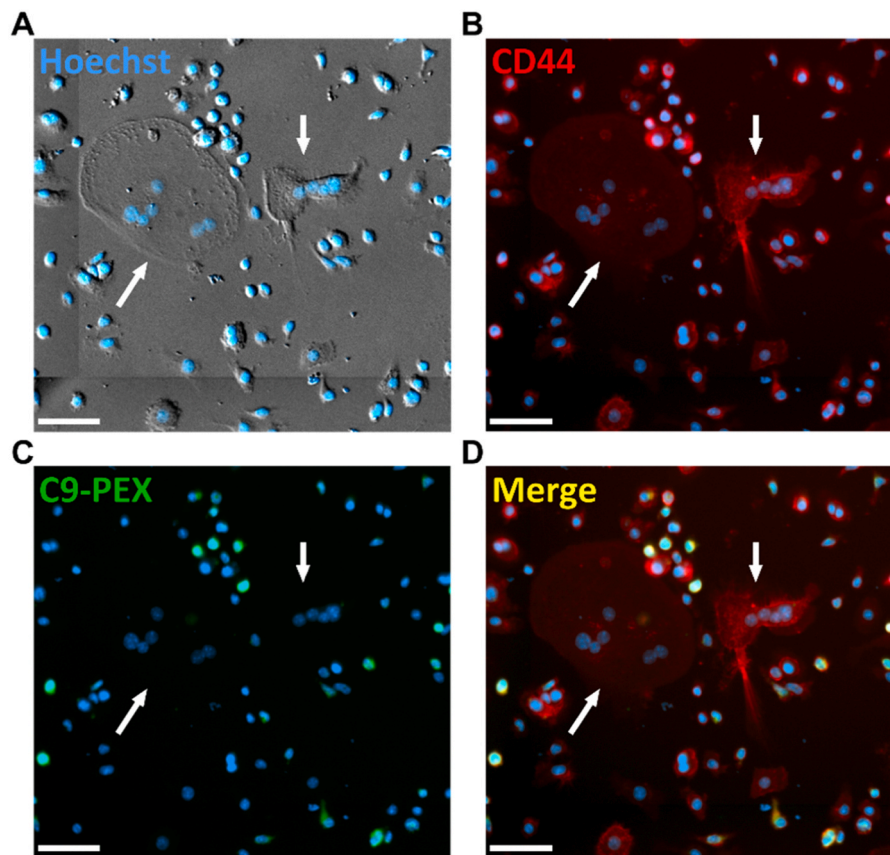


Fig. 7. Bright field and fluorescence microscopy images of heterogeneous mixture of OCs and differentiating CD14 + monocytes stained with: (A) Hoechst stain; (B) rat anti-CD44 antibody treated with secondary anti-rat antibody conjugated to Alexa Fluor 568 (red); and (C) C9-PEX treated with anti-c-myc antibody conjugated to FITC. (D) Merged image of panels B and C. Yellow color represents co-localization of C9-PEX and CD44. OCs are indicated with white arrows. Scale bar, 50 μm .

OCs, as previously reported in the literature [33]. To this end, effects on bone resorption from bone slices of OCs that had been allowed to differentiate from BMMs in the presence of C9-PEX, C9, A-C9-PEX and C9 +PEX were assessed. Our results showed that at a concentration of 250 nM, none of the proteins significantly inhibited bone resorption (Fig. 9). However, at ≥ 500 nM, both C9-PEX and A-C9-PEX, but not C9,

demonstrated significant inhibition compared to the untreated group. These findings indicate that C9-PEX and A-C9-PEX reduced OC-mediated bone resorption alongside their effect on OC formation, highlighting the role played by CD44 in OC formation and overall resorptive outcome (Fig. 9). Because resorption areas were not normalized to number of OCs on the same bone slices, decreased total

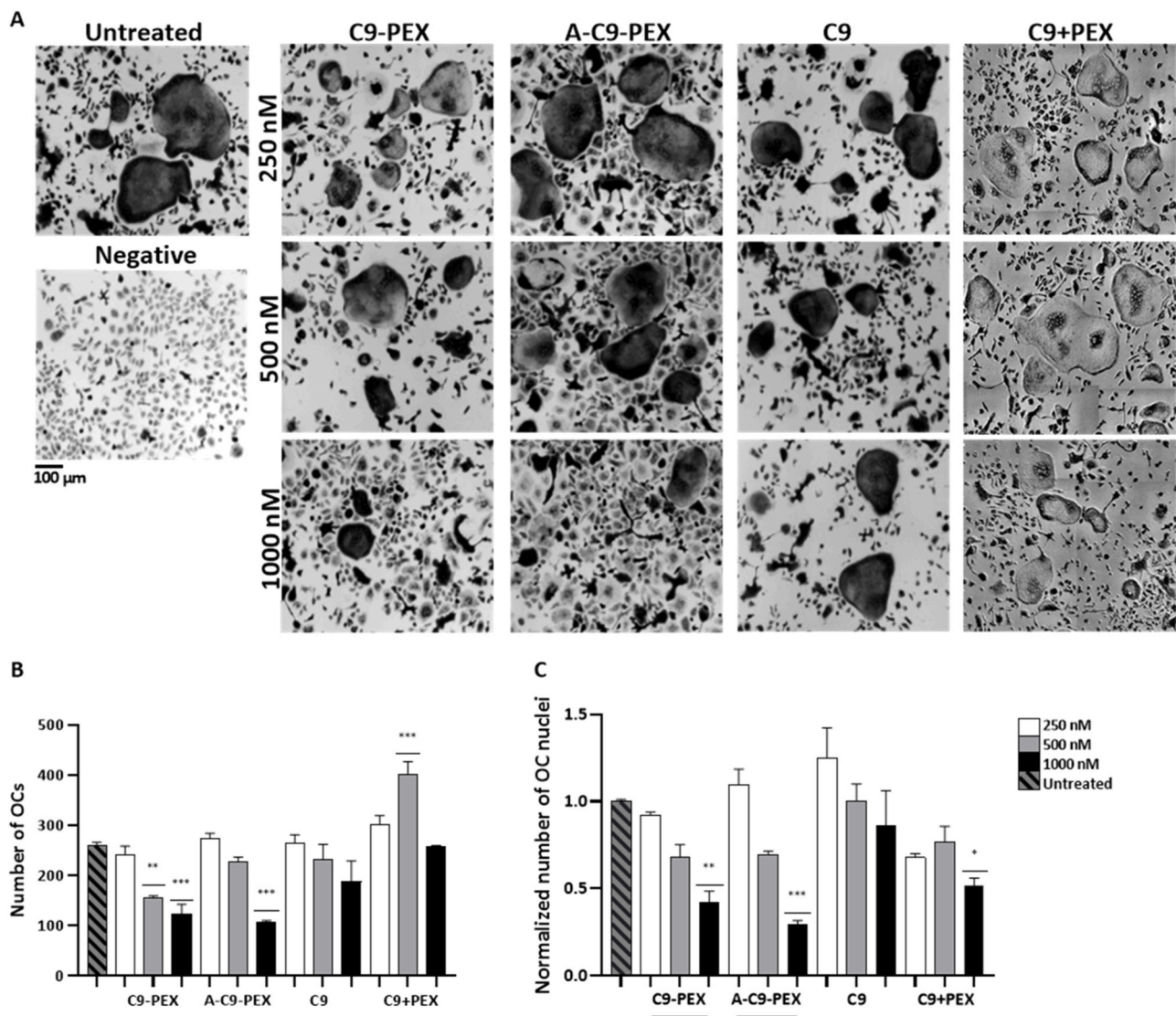


Fig. 8. OC differentiation using a medium with recombinant mouse M-CSF (20 ng/mL), RANKL (50 ng/mL), but with different concentrations of inhibitors. The same medium without inhibitors was used for the untreated group. The medium for the negative control was supplemented with recombinant M-CSF alone. (A) Representative images of each experimental group; scale bar, 100 μ m (indicated under the 'Negative' image). (B) Number of OCs. (C) Number of OC nuclei normalized to the untreated control group (n = 3). Error bars represent SEM. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, using one-way ANOVA followed by Dunnett's multiple-comparisons test for comparison with the untreated group. Graphs were plotted using GraphPad Prism 8.0.1.

resorption may reflect fewer OCs, reduced OC maturation, and/or reduced per-cell resorptive activity. Nevertheless, from a therapeutic perspective, the reduction in overall OC-mediated resorption remains functionally relevant and is consistent with the inhibitory effects observed in the OC differentiation assay. Nevertheless, because these experiments were performed in murine bone marrow-derived cultures, future studies using human OCs will be required to define the translational efficacy of C9-PEX and the exposure levels required for therapeutic activity.

Conclusions

This study shows that the chimera protein C9-PEX acts as a potent inhibitor of OC activity by targeting both a membrane-bound protein (CD44) and a soluble protein (MMP9). The chimera exhibits high affinity for CD44 and MMP9_{CAT} and inhibits the activities of both MMP9_{CAT} (in the picomolar range under our fluorogenic assay conditions) and MMP9 secreted by OCs, resulting in marked inhibition of OC differentiation and reduction of OC-mediated bone resorption in murine

bone marrow-derived OC cultures without detectable cytotoxic effects. At matched nominal concentrations, C9-PEX showed stronger inhibitory activity than the comparative control proteins and a 1:1 mixture of C9 and PEX, supporting a functional advantage of simultaneous target engagement within a single fusion format. Future studies using human OCs and pre-clinical models will be required to determine the translational efficacy of C9-PEX and define the exposure requirements needed for therapeutic activity. Although binding-site/valency-equivalent dosing and formal combination modeling were not applied here, the findings support C9-PEX as a promising approach for selective inhibition of OC function and hence a new pharmaceutical pathway for the development of an osteoporosis medication.

CRedit authorship contribution statement

Noam Levaot: Writing – review & editing, Visualization. **Shani Ohayon:** Writing – review & editing, Formal analysis, Data curation. **Dan Pasternak:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation.

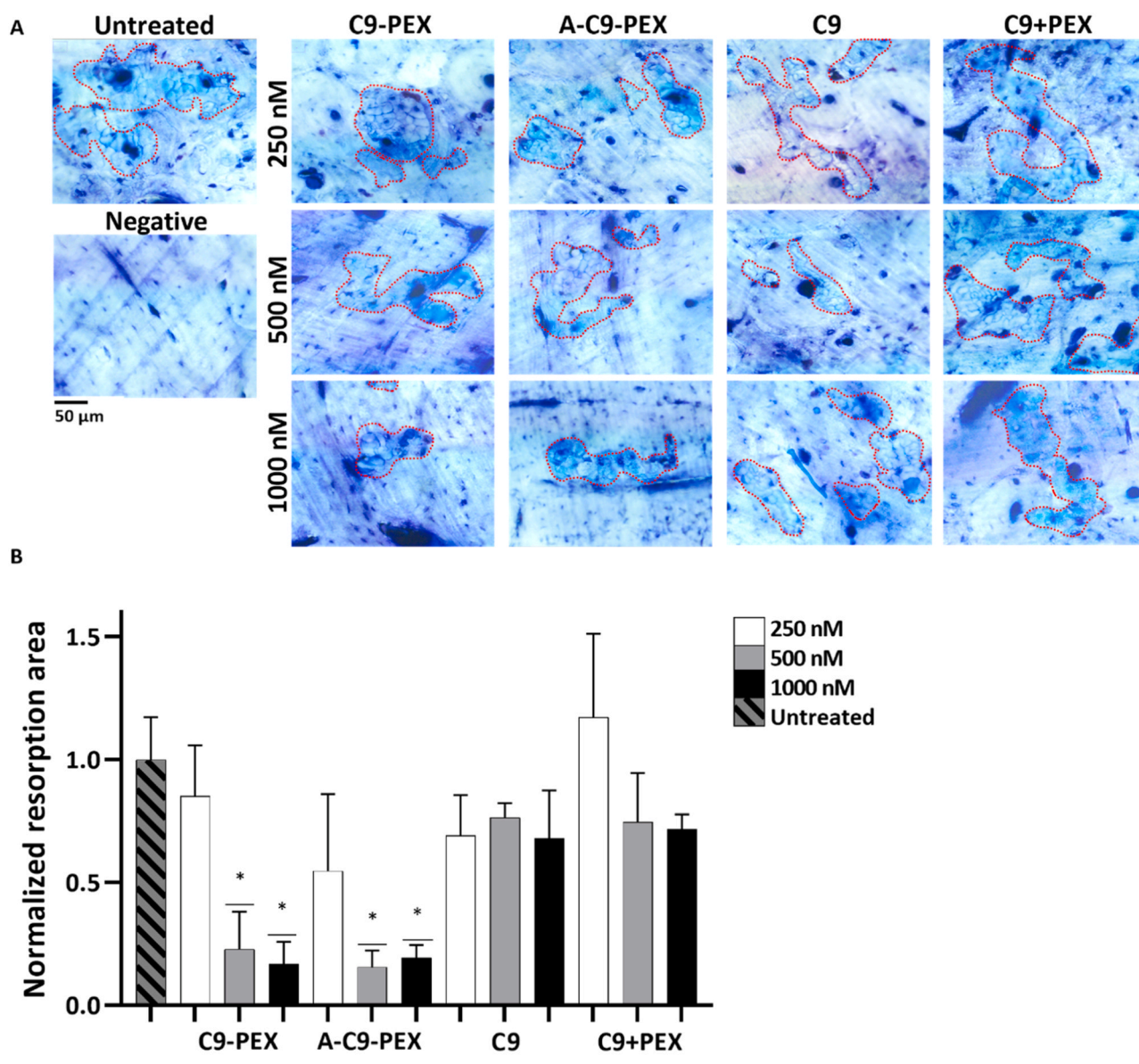


Fig. 9. Inhibition of bone resorption on bone slices. OCs were differentiated on bone slices using a medium with recombinant mouse M-CSF (20 ng/mL), RANKL (50 ng/mL), but with different concentrations of inhibitors. The same medium without inhibitors was used for the untreated group. The medium for the negative control was supplemented only with recombinant M-CSF. (A) Representative images of the different treatment groups showing the resorption areas on the bone surface, indicated with red dashed line. Scale bar, 50 μ m (indicated under the 'Negative' image). (B) Normalized resorption area relative to the untreated group (n = 3). Error bars represent SEM. * $P \leq 0.05$ using one-way ANOVA followed by Dunnett's multiple-comparisons test for comparison with the untreated group. Graph was plotted using GraphPad Prism 8.0.1.

Niv Papo: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Investigation, Funding acquisition, Conceptualization.

Funding

This work was supported by the National Institutes of Health (R01GM144393), the Rosetrees Trust (OoR2022/100004), the Israel Science Foundation (grant number 1615/19), and the Worldwide Cancer Research (grant number 20–0238) to NP.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence

the work reported in this paper.

Acknowledgments

The authors thank Dr. Alon Zilka for his technical assistance with SPR and MST experiments, and Dr. Alexandra Tsitrina and Dr. Uzi Hadad for their technical assistance with Zeiss Cell Discoverer 7 microscope.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.nbt.2026.05.002](https://doi.org/10.1016/j.nbt.2026.05.002).

Data availability

Data will be made available on request.

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