Glucose-dependent insulinotropic polypeptide (GIP) dose-dependently reduces osteoclast differentiation and resorption

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A role for glucose-dependent insulinotropic polypeptide (GIP) in controlling bone resorption has been suspected. However uncertainty remains to identify whether GIP act directly on osteoclasts. The aim of the present study were (i) to identify in different osteoclast differentiation models (human peripheral blood mononuclear cells-PBMC, murine bone marrow macrophage-BMM and murine Raw 264.7 cells) whether GIP was capable of reducing osteoclast formation and resorption; (ii) ascertain whether the highly potent GIP analogue N-AcGIP was capable of inducing a response at lower concentrations and (iii) to decipher the molecular mechanisms responsible for such effects. [D-Ala2]-GIP dose-dependently reduced osteoclast formation at concentration as low as 1 nM in human PBMC and 10 nM in murine BMM cultures. Furthermore, [D-Ala2]-GIP also reduced the extent of osteoclast resorption at concentration as low as 1 nM in human PBMC and murine BMM cultures. The mechanism of action of [D-Ala2]-GIP appeared to be mediated by reduction in intracellular calcium concentration and oscillation that subsequently inhibited calcineurin activity and NFATc1 nuclear translocation. The potency of the highly potent N-AcGIP was determined and highlighted an effect on osteoclast formation and resorption at concentration ten times lower than observed with [D-Ala2]-GIP in vitro. Furthermore, N-AcGIP was also capable of reducing the number of osteoclast in ovariectomized mice as well as the circulating level of type I collagen C-telopeptide. Pharmacological concentrations required for reducing osteoclast formation and resorption provide the impetus to design and exploit enzymatically stable GIP analogues for the treatment of bone resorption disorders in humans.

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1. Introduction

Osteoclasts are multinucleated cells which originate from the hematopoietic lineage (CFU-GM) and are capable of lacunar bone resorption [1,2]. Several factors have been demonstrated to modulate osteoclast formation and activity and the receptor activator of nuclear factor-κB (NF-κB) ligand (RANKL) seems to be crucial for osteoclast differentiation. Indeed, the binding of RANKL to its receptor, RANK, leads to a series of intracellular events that activates several downstream targets including p38 mitogen-activated protein kinase (p38), extracellular signal–regulated kinase (ERK), c-jun N-terminal kinase (JNK), NF-κB, Akt and nuclear factor of activated T-cells-1 (NFATc1) [3]. Nuclear factor of activated T-cells-1 is crucial for terminal osteoclast differentiation as NFATc1-deficient embryonic stem cells fail to differentiate into osteoclasts, whilst ectopic expression of NFATc1 causes precursor cells to differentiate into multinucleated osteoclasts [4]. The distinguishing feature of NFATc1 is its regulation by Ca2⁺ and the Ca2⁺/calmodulin-dependent serine phosphatase calcineurin. NFATc1 is phosphorylated and sequestered in the cytoplasm of resting cells but upon stimulation, it becomes dephosphorylated by calcineurin, translocates to the nucleus, and becomes transcriptionally active [5].

Bone remodeling is a normal physiological process that involves the resorption of bone by osteoclasts and the synthesis of bone matrix by osteoblasts. Bone remodeling is tightly controlled by a complex network of hormonal, neuronal and local factors as well as by cell-to-cell contact [6]. Among all these factors, a potentially important role for gut hormones to control osteoclast activity has been postulated [7,8]. Indeed, bone remodeling is reduced after parenteral feeding [9], and the pattern of bone resorption is rapidly reduced after a meal [10]. Moreover, food fractionation is capable of altering bone resorption and increasing bone mineral density to a much greater extent than observed with a matched nutrient load given once a day [11].
Of all the gut hormones, glucose-dependent insulinotropic polypeptide (GIP), also known as gastric inhibitory polypeptide, represents a particularly interesting candidate regulator of bone function. GIP is synthesized by K-cells of the upper small intestine and is rapidly secreted into the blood stream after nutrient enter the intestinal lumen [12]. Intravenous administration of GIP in healthy volunteers resulted in a 49% decrease in the circulating resorption marker c-terminal collagen crosslinks (CTX), suggesting possible anti-resorptive actions of GIP [13]. However, inconsistent data on the actions of endogenous GIP on osteocyte physiology have been reported from various strains of GIP receptor (GIPr) knock-out mice [14–16]. Indeed, in GIPr-deficient animals either no modification of osteocyte number and resorptive capacity, increased osteocyte number and resorption or reduced osteocyte number and resorption have been reported [14–16]. Experiments with these models provide no information on whether GIP acts directly or indirectly on osteoclast precursors and/or mature osteoclast to alter their differentiation and activity. Zhong et al. reported the presence of a functional GIP in multinucleated osteoclasts [17]. Furthermore, GIP was shown to decrease PTH-mediated osteocyte resorption in a fetal rat long bone resorption assay, which is a co-culture system with osteoclast precursors, mature osteocytes, osteoblasts and stromal cells [17]. However, although this evidence suggests that the GIPr is expressed in osteoclasts, use of the fetal rat long bone resorption assay does not answer the question of whether the observed reduction in bone resorption was due to a direct action of GIP on osteoclasts. Another unresolved question is whether long-acting GIP analogues, that show high potency at the receptor (e.g. N-AcGIP), have more pronounced effects than native or [D-Ala2]-GIP at reducing bone resorption.

To further delineate the actions of GIP on osteoclasts, the present study aims to (i) ascertain whether GIP controls osteoclast formation and resorption directly using different osteoclast assays (human peripheral blood mononuclear cells-PMBCs, bone marrow macrophages-BMM, murine Raw 264.7 cells), (ii) investigate whether N-AcGIP is more effective at reducing osteoclast resorption and (iii) to decipher the mechanisms by which GIP controls osteoclast differentiation.

2. Material and methods

2.1. Reagents

Alpha-Minimum Essential Medium Eagle (α-MEM), heat-inactivated fetal bovine serum (FBS), penicillin and streptomycin were purchased from Lonza (Levallois-Perret, France). Macro-phase-colony stimulating factor (M-CSF) and soluble receptor activator of nuclear factor kappa B ligand (sRANKL) were purchased from R&D Systems Europe (Abingdon, UK) and Peprotech Ltd (London, UK), respectively. All other chemicals were purchased from Sigma-Aldrich (Lyon, France) unless otherwise stated.

2.2. Peptide synthesis

GIP analogues with a d-alanine in position 2 ([D-Ala2]GIP) or an acetyl adduct incorporated at the N-terminal Tyr1 (N-AcGIP) were purchased from Genecust (Dudelange, Luxembourg) and EZBiolabs (Carmel, IN), respectively. Both peptides were synthesized using standard solid-phase Fmoc peptide chemistry. Substitution of alanine at the N-terminal position 2 with its (D) isomer or acetylation of Tyr1 was performed to enhance resistance to serum dipeptidylpeptidase-4 as described elsewhere [18]. Peptide purity was evaluated by reverse-phase HPLC (purity > 95%) and electrospray ionization mass spectroscopy (ESI-MS). Peptide sequences and characteristics are reported in Table 1. Peptides were dissolved in phosphate buffer saline at a concentration of 10–5 M, aliquoted and stored at –80 °C until use.

2.3. Isolation and osteoclast generation from human peripheral blood mononuclear cells (PBMCs)

PBMCs were isolated from 5 buffy coats obtained at the Etablissement Français du Sang (Angers, France) as described previously [19]. Blood was diluted 1:1 in α-minimal essential medium (MEM), layered over Histopaque and centrifuged (700 × g) for 20 min. The interface layer was resuspended in MEM then centrifuged (600 × g) for a further 10 min after which the resultant cells were resuspended in media supplemented with 10% FCS and counted in a hemocytometer following lysis of red blood cells using a 5% (v/v) acetic acid solution. To assess the extent of osteoclast formation and activation, isolated human PBMCs were cultured either in 24-well plates or collagen-coated 24 wall plates at a concentration of 2 × 105 PBMCs/ml in MEM containing 100 U/ml penicillin, 100 μg/ml streptomycin and 10% FCS (osteoclast medium) [20]. After 2 h incubation, cultures were vigorously rinsed in medium to remove non-adherent cells, and then maintained in 1 ml MEM/FCS with 25 ng/ml recombinant human M-CSF, 100 ng/ml recombinant human sRANKL (added at day 7) and various concentrations of GIP analogues (added at day 7). Cultures were terminated after 14 days to assess the extent of osteoclast formation (TRAP staining as described below) or at 20 days to assess the extent of osteoclast resorption (see below). All factors were replenished every 2–3 days.

2.4. Isolation and osteoclast generation from murine bone marrow macrophage (BMM)

Bone marrow macrophages were isolated from the long bones of 6 week-old male BALB/c mice (strain BALB/cJrj) by flushing tibias and femurs with alpha-MEM as previously reported [21]. This experimental protocol has been approved by the Institutional Animal Care and Use Committee at the University of Angers. Bone marrow cells were cultured for 24 h in a 25 cm2 flask in alpha-MEM supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin to facilitate stromal cells attachment. Non adherent cells were then collected and plated at a concentration of 2 × 106 cells/ml in either 24-well plates or collagen-coated 24 well plates in alpha-MEM supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 25 ng/ml M-CSF, 50 ng/ml sRANKL and several concentrations of GIP analogues (added at day 7). Cultures were terminated after 14 days to assess the extent of osteoclast formation (TRACP staining as described below) or at 20 days to assess the extent of osteoclast resorption (see below). All factors were replenished every 2–3 days.

2.5. Osteoclast generation from murine Raw 264.7 cells

Murine Raw 264.7 cells (Clone TIB-71) were purchased from the American type culture collection (ATCC, Molsheim, France). In order to generate osteoclasts, Raw 264.7 cells were plated at a concentration

Table 1

| Characteristics of GIP analogues. |
|-------------------------------|-----------------|----------------|
| Peptide          | Sequence         | In vitro DPP-4 resistance (h) | cAMP production EC50 |
| GIP              | YAEGTFSYSSMDKHQQDFYVVNLALQKKGNDWKNITQ | 2.2 | 18.2 nM |
| [D-Ala2]GIP      | Y[D-Ala]EGTFSSYSDKHQQDFYVVNLALQKKGNDWKNITQ | >24 | 10.2 nM |
| N-AcGIP         | N-Acetyl-YAEGTFSYSSMDKHQQDFYVVNLALQKKGNDWKNITQ | >24 | 9.4 nM |

For DPP-4 resistance studies, peptides were incubated with murine plasma for 0, 2, 4, 8 and 24 h and reaction products examined by HPLC with degradation calculated as percentage intact peptide remaining (n = 3). For cAMP assays, BRIN-BD11 cells were exposed to various concentrations of GIP or related analogues (10−12 to 10−6 M). All test incubations were performed for 20 min and cAMP measured by ELISA (n = 4).
of 2.5 × 10^5 cells/ml in 24-well plate in alpha-MEM supplemented with 10% FCS, 2 mM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 100 ng/ml sRANKL and several concentrations of GIP analogues. After seven days of culture, TRAPc staining was performed as described below to evidence the presence of osteoclast cells. All factors were replenished every 2–3 days.

2.6. Characterization of newly-formed osteoclasts

Expression of the tartrate-resistant acid phosphatase (TRAPc) was examined cytochemically, as described previously [22]. Briefly, cells were rinsed promptly in PBS buffer, fixed with formalin (10% in PBS buffer) for 10 min and rinsed in distilled water. TRAPc was cytochemically demonstrated by a simultaneous coupling reaction using Naphtol AS-Bl-phosphate as substrate and Fast violet B as the diazonium salt. Cells were then incubated for 90 min at 37 °C in the dark, rinsed three times in distilled water and the residual activity was inhibited by 4% NaF for 30 min. Cells were then rinsed in distilled water, counterstained with 4, 6-diamidino-2-phenylindole (DAPI) for 20 min and allowed to dry. TRAPc positive cells, with more than three nuclei, were identified as osteoclasts.

2.7. Characterization of bone resorption

At day 7 (BMMs) or day 20 (PBMCs), cells cultured on collagen-coated plates were incubated with collagenase for 30 min at 37 °C. The cell suspension was passed through a cell strainer with 40-μm pores (BD Falcon, Erembodegem, Belgium), and the fraction retained in the strainer was carefully detached by inverting the filter and rinsing with culture medium. Cells were counted and 2 × 10^6 cells were plated on dentine slices and cultured for an additional 24 h. The dentine slices were removed from the culture wells, placed in NH4OH (1 N) for 30 min and sonicated for 5 min to remove adherent cells. After rinsing in distilled water, the dentine slices were stained with 0.5% (v/v) toluidine blue (pH 5.0) prior to examination by light microscopy. The surface of each dentine slice was examined for the presence of lacunar resorption, the extent of surface erosion on dentine slices was determined using image analysis as described previously [19].

2.8. Animal study

Fourteen ovariectomized female BALB/c mice (strain BALB/cJrj) were obtained from Janvier Labs (Saint-Berthevin, France). The BALB/c strain was chosen because these mice exhibit deterioration of trabecular and cortical microstructure in response to ovariectomy [23]. Bilateral ovariectomy was performed on 12-week-old mice by a veterinary surgeon at the animal supplier farm. Briefly, general anesthesia was induced by intraperitoneal injection of a mix of ketamine (Imalgene 1000®, 100 mg/kg) and xylazine (Rompun 2%®, 10 mg/kg) supplemented with subcutaneous injection of the pain killer carprofen (Rimadyl®, 5 mg/kg). Skin and muscle incisions were made on the lateral side of the animal and the ovaries were ligatured and sectioned. Incisions were then closed by surgical sutures. All animals were housed in a temperature- and humidity-controlled room providing a 12-hour light: 12-hour dark cycle with free access to standard rodent chow and water. At 16 weeks of age, two groups of ovariectomized (OVX) animals were studied and injected subcutaneously with: vehicle daily (normal saline) (OVX + Veh animals, n = 8) or 25 nmol/kg/day b.w. N-AcGIP (OVX + N-AcGIP, n = 6). This dosing regimen for the GIP analogue was based on previous published studies where this molecule was proven active with beneficial effects on bone [24]. Ten Sham-operated female BALB/c mice (mouse strain BALB/cJrj, Janvier Labs) with the same age and injected daily with saline were used as controls. Blood samples were collected by intracardiac aspiration, spun at 800 × g for 15 min, and sera stored at −20 °C until analysis. After necropsy, tibias were collected and cleaned of soft tissue. All animal experiments were approved by the regional ethical committee (CEEA-Pdl06-01740.01) and the Institutional Animal Care and Use Committee at the University of Angers. All procedures were conducted according to the Principles of laboratory animal care (NIH publication no. 85-23, revised 1985) and the French Animal Scientific Procedures Act 2013-118.

2.9. Microcomputed X-ray tomography (MicroCT)

MicroCT analysis was performed with a Skyscan 1172 microtomograph (Bruker MicroCT, Kontich, Belgium) operated at 70 kV, 100 μA, 340-ms integration time. The isotropic pixel size was fixed at 3.75 μm, the rotation step at 0.25° and exposure was done with a 0.5-mm aluminum filter. Each 3D reconstruction image dataset was binarized using global thresholding. Trabecular parameters were assessed in the proximal tibia metaphysis 0.5 mm below the growth plate and extending 2 mm down with the CTan software (Bruker MicroCT). Cortical parameters were assessed 4 mm below the growth plate and extended 1 mm down in the tibia diaphysis. All parameters were determined according to guidelines and nomenclature proposed by the American Society for Bone and Mineral Research [25].

2.10. Three point bending

Three-point bending experiments were performed on femurs as reported previously [26]. Prior to mechanical testing, femurs were rehydrated in saline for 24 h at 4 °C. Measurements were done with a constant span length of 10 mm on an Instron 5942 (Instron, Elancourt, France). Femurs were positioned horizontally with the anterior surface facing downward, centred on the support and the pressing force was applied vertically to the midshaft of the bone. The load-displacement curve was acquired with the Bluehill 3 software (Instron). Maximum load and stiffness were computerized according to previously published equations [27].

2.11. Bone histomorphometry

After microCT scans, tibias were dehydrated in three successive baths of acetone followed by a bath in xylene (24 h each) at 4 °C. Impregnation was made by three baths (12 h each) in methylmethacrylate at 4 °C. Embedding of undecalcified specimen in poly(methylmethacrylate (PMMA) was then carried out at 4 °C in order to preserve TRAPc activity. For each animal, four non serial sections (~50 μm apart) were stained for the osteoclastic TRAPc using an naphthyl phosphate and fast violet B as previously described in details [28]. Only TRAPc-positive nucleated cells in contact with bone were counted as osteoclasts. Regions of interest (ROI) were positioned at the same locations as microCT ROIs. Standard bone histomorphometry nomenclatures, symbol and units were used as described in the guidelines of the American Society for Bone and Mineral Research [29]. The identity of the section was not revealed until the end of all measurements.

2.12. CTx

Levels of C-terminal type I collagen cross-links (CTx-II) were measured in non-hemolyzed sera by ELISA, as described by the manufacturer (Ratlaps, IDS Ltd, Bolton, UK).

2.13. Immunofluorescence staining

Raw 264.7 cells were seeded at a concentration of 2 × 10^4 cells/cm² on glass coverslips and treated with sRANKL (100 ng/ml) alone or in combination with (D-Ala²)GIP (100 nM) for 48 h. Then, cells were fixed with 3.7% formaldehyde in saline for 15 min and permeabilized with 0.05% triton X100 for 2 min. Cells were sequentially incubated in 5% BSA (bovine serum albumin) in Tris 0.1 M buffer for 1 h, 2 μg/ml
mouse anti-NFATc1 monoclonal antibody (sc-7294, Santa Cruz Biotechnology Inc., Heidelberg, Germany) in 1% BSA-containing Tris buffer for 3 h, and then 5 μg/ml anti-mouse IgG-NL557 antibody (R&D Systems Europe, Abingdon, UK). Nuclear staining was acquired by adding DAPI for 20 min at the end of NFATc1 labelling procedure. Samples were imaged with a Leica TCS SP8 confocal microscope (Leica Microsystems SAS, Nanterre, France). At least five different fields of the glass coverslips were images. Co-localization of both probes was determined by creating region of interest around cells and by computing the Pearson’s correlation coefficient (PCC) calculated as [30]:

\[
PCC = \frac{\sum_{i} (R_i - \bar{R}) \times (G_i - \bar{G})}{\sqrt{\sum_{i} (R_i - \bar{R})^2 \times \sum_{i} (G_i - \bar{G})^2}}
\]

where Ri and Gi refer to the intensity values of the DAPI and NFATc1 channels, respectively, of pixel i, and R and G refer to the mean intensities of the DAPI and NFATc1 channels, respectively, across the region of interest. This approach is simple, robust and reproducible and quantifies the degree to which the variability in both fluorescent channel intensities can be explained with a simple, linear relationship between the two. The absence of co-occurrence of both probes would result with a PCC tending to 0 whilst a high positive occurrence of both probes would result in PCC tending to 1. This approach appeared superior to the Mander’s overlap coefficient [31].

2.14. Förster Resonance Energy Transfer (FRET) analysis

Raw 264.7 cells were plated at a concentration of $2 \times 10^4$ cells/cm² in a 4-well microslides (ibidi GmbH, Martinsried, Germany). Twenty-four

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**Fig. 1.** Osteoclast formation and resorption in human peripheral blood mononuclear cells (PBMC) and murine bone marrow macrophage (BMM) cultures exposed to (D-Ala²)GIP. Osteoclast precursors were cultured in the presence of M-CSF, M-CSF + RANKL or M-CSF + RANKL plus (D-Ala²)GIP (100 pM, 1 nM, 10 nM and 100 nM). The total numbers of newly-formed multinucleated TRAcP positive cells, as well as the extent of lacunar bone resorption, were significantly and dose-dependently lower in the presence of (D-Ala²)GIP. M: M-CSF, MR: M-CSF + RANKL, *: p < 0.05 and **: p < 0.01 vs. MR; #: p < 0.05 and ##: p < 0.01 vs. previous concentration of (D-Ala²)GIP. Scale bar = 100 μm.
hours later, cells were transfected with 1 μg plasmid encoding the mTurquoise-EPAC-cp173Venus-Venus probe. kindly provided by Professor K. Jalink (Netherland Cancer Institute, Amsterdam, Netherland), using Lipofectamine 3000 as described by the manufacturer (Life technologies, Saint-Aubin, France). Experiments were performed in HEPES buffered saline (containing 140 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 10 mM glucose, 10 mM HEPES) in a chamber containing a Leica imaging system (DMi6000 Inverted microscope fitted with a SP8 confocal head) and a controlled atmosphere (37 °C, 5% CO₂). The image acquisition was performed with a 63×, 1.4 N.A. oil immersion objective and a Hybrid® detector (Leica) and images were collected every 5 s over a period of 30 min. Donor excitation was made with a 458 nm Ar laser, donor emission was collected between 460 and 505 nm and acceptor emission between 520 and 600 nm by setting the SP8 spectrometer accordingly. FRET was expressed as the ratio between donor and acceptor signals. The FRET value was set at 1 at the onset of the experiment. Cells were stimulated with 100 nM (D-Ala²)GIP or 25 μM forskolin. A minimum of 30 cells were imaged per condition.

2.17. Statistical analysis

Each in vitro experiment has been replicated at least three times. Results were expressed as mean ± standard error of the mean (SEM). Non-parametric Kruskal-Wallis and Mann-Whitney U test were used to compare the differences between the groups using the Systat statistical software release 13.0 (Systat software Inc., San Jose, CA). Differences at p < 0.05 were considered to be significant.

3. Results

3.1. (D-Ala²)GIP directly inhibits osteoclast formation and resorption

As shown in Fig. 1, osteoclasts were generated in the presence of M-CSF and sRANKL from human PBMCs. The addition of (D-Ala²)GIP in these cultures dose-dependently reduced osteoclast formation with significant 12%, 24% and 36% reductions observed respectively, in the presence of 1 nM, 10 nM and 100 nM (D-Ala²)GIP. However, the lower (D-Ala²)GIP concentration had no effect on osteoclast formation. The extent of osteoclast resorption was also significantly reduced by 60% and 84% in PBMCs cultures at 10 nM and 100 nM (D-Ala²)GIP, respectively. Lower concentrations of (D-Ala²)GIP did not significantly reduce osteoclast resorption in PBMCs cultures. The extent of osteoclast formation and resorption was also assessed in BMMs cultures. Similarly, 1 nM, 10 nM and 100 nM (D-Ala²)GIP significantly decreased the number of osteoclasts by 21%, 34% or 44%, respectively. Lower (D-Ala²)GIP concentrations had no effect on osteoclast formation. Osteoclast resorption was also substantially altered in the presence of (D-Ala²)GIP concentrations.
higher than 100 pM, with significant reductions of 28%, 45% and 66% being observed with doses of 1 nM, 10 nM and 100 nm, respectively.

3.2. (D-Ala²)GIP blocks the nuclear translocation of NFATc1

In order to decipher how GIP reduces osteoclast differentiation, we looked at the nuclear translocation of NFATc1 (Fig. 2). As revealed in Fig. 2A, after 48 h of stimulation with 100 ng/ml RANKL, a significant amount of NFATc1 was translocated into the nucleus. Co-localization studies revealed a strong co-occurrence between DAPI and NFATc1 as demonstrated by the Pearson’s correlation coefficient. On the other hand, co-treatment with 100 ng/ml RANKL and 100 nM (D-Ala²)GIP for 48 h led to a sequestration of NFATc1 into the cytoplasm. Under these circumstances, the Pearson’s correlation coefficient was significantly lower in (D-Ala²)GIP treated cultures \( p < 0.01 \), suggesting lower co-localization between NFATc1 and DAPI and hence confirming the sequestration of NFATc1 into the cytoplasm.

3.3. (D-Ala²)GIP reduces osteoclast differentiation by a pathway independent of the adenylyl cyclase-cAMP-Protein Kinase A pathway

To ascertain the mechanisms involved in NFATc1 sequestration in the cytoplasm, we examined the extent of cAMP release in osteoclast precursor cultures. As shown in Fig. 3B, surprisingly (D-Ala²)GIP
treatment did not increase intracellular cAMP. To verify that our system was capable of detecting an increase of intracellular cAMP, we treated parallel cultures with 25 μM forskolin, which resulted in a marked augmentation of cAMP. The involvement of the adenylyl cyclase pathway can also be dissected using pharmacological inhibitors as depicted in Fig. 3A. The number of newly-formed osteoclasts generated in the presence of RANKL and (d-Ala²)GIP was unaffected by the presence of IBMX, 2',5' DDA or H89 (Fig. 3C) suggesting that the conventional adenylyl cyclase-cAMP-PKA pathway was not involved in this osteoclast response to GIP.

3.4. (d-Ala²)GIP reduces osteoclast differentiation by reducing rise in intracellular calcium [Ca²⁺].

Another important contributor generating osteoclast and NFATc1 nuclear translocation is the rise of [Ca²⁺]i following RANKL stimulation. We therefore assessed whether RANKL stimulation could increase [Ca²⁺]i. As shown in Fig. 4A, [Ca²⁺]i was increased rapidly after RANKL administration. However, when (d-Ala²)GIP was added to the osteoclast precursor cultures, only a modest rise in [Ca²⁺]i (approximately twice less than observed with RANKL) was recorded. More

![Graph showing intracellular calcium percentage positive control over time.](A)

![Graph showing AUC percentage second.](B)

![Graph showing frequency calcium spike.](C)

**Fig. 4.** (d-Ala²)GIP blocks the nuclear translocation of NFATc1 by reducing intracellular Ca²⁺ rises. (A) RANKL (dark circles), (d-Ala²)GIP (white circles) and RANKL+(d-Ala²)GIP (grey circles) have been injected and the [Ca²⁺]i were determined using the Fluo-4 probe over time. The arrow indicates when drugs were administered. (B) [Ca²⁺]i oscillations were recorded 48 h after administration of RANKL or RANKL+(d-Ala²)GIP and the frequency of Ca²⁺ spikes was determined. (C) Calcineurin activity was assessed with the malachite green method in the presence of saline vehicle, RANKL or RANKL plus (d-Ala²)GIP. **: p < 0.01 vs. RANKL-treated cultures. Veh: vehicle, AUC: Area under the curve, RFU: Relative fluorescence unit.
importantly, the co-addition of RANKL and (D-Ala²)GIP led to a 54% reduction of [Ca²⁺], as compared with RANKL alone (p < 0.01). Furthermore, Ca²⁺ free buffer totally abolished the RANKL-mediated augmentation of [Ca²⁺], suggesting that this phenomenon was due to Ca²⁺ entry (data not shown).

Another important feature of RANKL-induced osteoclast formation is the induction of spontaneous Ca²⁺ oscillations after few days of culture. As shown in Fig. 4B, RANKL-treated cultures exhibited Ca²⁺ oscillations with a frequency of ~1 oscillation per minute. On the other hand, RANKL plus (D-Ala²)GIP-treated cultures exhibited a decrease in the firing rate of Ca²⁺ oscillations with ~1 oscillation per 3 min (Fig. 4B). We next examined whether the downstream calcineurin was affected by reduction in this Ca²⁺ firing rate. Indeed, in the presence of RANKL, calcineurin activity was increased by 45% as compared with control cultures (p < 0.01). However, co-treatment of RANKL with (D-Ala²)GIP led to a significant reduction into calcineurin activity by 15% as compared with RANKL alone (p < 0.01).

3.5. N-AcGIP is a particularly potent activator of the GIPr for reduction of osteoclast formation and resorption

As reported previously, (D-Ala²)GIP has a prolonged half-life with the same potency at the GIPr as native GIP [33]. N-AcGIP, another GIP analogue with acetylation of the N-terminal Tyr¹ of GIP, is also resistant to protease degradation but with a greater potency at the GIPr compared with native GIP [34]. We wished therefore to ascertain whether N-AcGIP could reduce osteoclast formation and/or resorption at lower concentrations. As shown in Fig. 5, N-AcGIP was capable of reducing osteoclast formation by 14% at a concentration as low as 100 pM in PBMCs cultures in vitro. Indeed, this effect was dose-dependent as demonstrated by 31%, 42% and 54% reductions of osteoclast numbers in the presence of 1 nM, 10 nM and 100 nM N-AcGIP, respectively (Fig. 5). Furthermore, reduction of osteoclast resorption occurred at a lower concentration of N-AcGIP as compared with (D-Ala²)GIP, with a significant effect being observed at 1 nM.

3.6. Protease resistant N-AcGIP inhibits osteoclast formation and resorption in vivo

Following on from positive in vitro data, we wanted to assess whether the most potent stable GIP agonist, N-AcGIP, was capable of reducing osteoclast formation and resorption in vivo. We used the OVX mice as a model of high osteoclast activity. As depicted in Fig. 6A, OVX mice exhibited a lower trabecular bone mass. Administration of N-AcGIP to these animals suggested a slight increase of trabecular bone mass. As shown in Table 2, OVX mice exhibited a significant 27% decrease of BV/TV as compared with control animals, whilst administration of N-AcGIP to OVX mice significantly prevented the marked decrease in this parameter (−9%, p = 0.02). This improvement in bone mass was accompanied by modifications of trabecular microarchitecture. Indeed, trabecular number and trabecular separation were significantly reduced and elevated, respectively, in OVX mouse (−27%, p < 0.01 and 15%, p < 0.01) as compared with control animals. On the other hand, administration of N-AcGIP to OVX mice led to a non-significant augmentation in Tb.N

![Fig. 5. Osteoclast formation and resorption in human PBMCs exposed to N-AcGIP. Osteoclast precursors were cultured in the presence of M-CSF, M-CSF + RANKL or M-CSF + RANKL plus N-AcGIP (100 pM, 1 nM, 10 nM and 100 nM). The total number of newly-formed multinucleated TRAcP positive cells, as well as the extent of lacunar bone resorption, were significantly and dose-dependently lower in the presence of N-AcGIP. M: M-CSF, MR: M-CSF + RANKL, *: p < 0.05 vs. MR; #: p < 0.05 vs. previous concentration of N-AcGIP. Scale bar = 100 μm.](image-url)
mechanical competence of in ovariectomized mice (Table 2). We next were evidenced (Table 2). However, N-AcGIP treatment improved bio-
reversed in the presence of N-AcGIP although trends to improvement were evidenced (Table 2). However, N-AcGIP treatment improved bio-
mechanical competence of in ovariectomized mice (Table 2). We next investigated whether N-AcGIP was capable of reducing osteoclast numbers and osteoclast resorption in OVX animals. As expected, saline-treated OVX animals exhibited significant augmentations in N.Oc/B.Pm (52%, p = 0.01), Oc.S/BS (14%, p = 0.03) and serum CTx (121%, p = 0.002). In contrast, administration of N-AcGIP to OVX mice led to
reductions in N.Oc/B.Pm (−38%, p = 0.01), Oc.S/BS (−39%, p = 0.009) and serum CTx (−20%, p = 0.002) (Fig. 6B–D).

4. Discussion

Bone remodeling follows a circadian pattern with bone resorption elevated early morning [35]. Bone resorption is dramatically reduced after a meal and coincide with the maximum gut hormone secretion [10] suggesting a gut-to-bone connection. This gut-to-bone connection has been demonstrated clearly in animal models lacking either the glu-
cagon-like peptide-1 (GLP-1) or GIP receptors. Indeed, GLP-1 receptor or GIP receptor knock-out mice exhibit a profound reduction in bone strength with marked alterations in bone microarchitecture and tissue material properties [14,36,37]. Co-ablation of GIP and GLP-1 receptors led to additive alterations of bone strength [26]. However, little is known about the mechanisms of action of these gut hormones and whether these bone alterations represent a direct effect on bone cells. Previously, we reported that administration of GIP to osteoblast cultures directly improves the ability of these cells to increase enzymatic collagen cross-linking as well as the diameter of collagen fibers by a direct cAMP-dependent mechanism [38]. Several previous published studies have reported a role for GIP and its receptor in reducing bone resorption [10,13,17]. However, there is no clear information on whether this is due to a direct or indirect action on osteoclast cells. In the present study, we demonstrate that (D-Ala²)GIP directly reduced osteoclast for-
mation involving blockade of [Ca²⁺]i elevation and resultant sequestra-
tion of transcription factor NFATc1 in the cytoplasm.

(A) (B) (C) (D)

Table 2

<table>
<thead>
<tr>
<th>Microarchitecture parameters</th>
<th>Sham (n = 10)</th>
<th>OVX + saline (n = 8)</th>
<th>OVX + N-AcGIP (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BV/TV (%)</td>
<td>7.8 ± 0.3</td>
<td>5.7 ± 0.5*</td>
<td>7.1 ± 0.1**</td>
</tr>
<tr>
<td>Tb.Th (μm)</td>
<td>50.9 ± 1.0</td>
<td>48.8 ± 1.0</td>
<td>53.3 ± 1.1</td>
</tr>
<tr>
<td>Tb.N (mm)</td>
<td>1.53 ± 0.06</td>
<td>1.12 ± 0.09*</td>
<td>1.33 ± 0.04*</td>
</tr>
<tr>
<td>Tb.Sp (μm)</td>
<td>339 ± 7</td>
<td>390 ± 11*</td>
<td>364 ± 12**</td>
</tr>
<tr>
<td>Tr.Ar (mm²)</td>
<td>3.4 ± 0.10</td>
<td>3.8 ± 0.06*</td>
<td>3.6 ± 0.15</td>
</tr>
<tr>
<td>Ct.Ar (mm²)</td>
<td>0.86 ± 0.01</td>
<td>0.82 ± 0.02</td>
<td>0.83 ± 0.04</td>
</tr>
<tr>
<td>Mx.Ar (mm²)</td>
<td>2.6 ± 0.09</td>
<td>3.0 ± 0.06*</td>
<td>2.7 ± 0.12</td>
</tr>
<tr>
<td>Clt.Ar/Tt.Ar (%)</td>
<td>25.3 ± 0.6</td>
<td>21.5 ± 0.4*</td>
<td>23.3 ± 0.8</td>
</tr>
<tr>
<td>Ct.Th (μm)</td>
<td>162 ± 1</td>
<td>147 ± 3*</td>
<td>150 ± 2*</td>
</tr>
<tr>
<td>Maximum load (N)</td>
<td>18.6 ± 0.4</td>
<td>14.0 ± 0.4*</td>
<td>17.1 ± 0.6*</td>
</tr>
<tr>
<td>Stiffness (N/mm)</td>
<td>92.0 ± 2.8</td>
<td>68.6 ± 3.4*</td>
<td>84.0 ± 3.6*</td>
</tr>
</tbody>
</table>

Microarchitecture parameters were determined by microCT with a voxel size of 3.7 μm.

- p < 0.05 vs. Sham.
- p < 0.05 vs. OVX + saline.
Due to the susceptibility of native GIP to enzymatic degradation by DPP-4 in vivo and in plasma, we utilized two stable analogues of GIP to evaluate the effect of GIPr activation [33,34]. The minimal concentration required to reduce osteoclast differentiation in the present study was 0.1 nM with N-AcGIP and 1 nM with (D-Ala²)GIP. Despite both analogues being resistant to DPP-4 degradation, the potency to activate the GIPr was stronger for N-AcGIP than (D-Ala²)GIP [33,34]. This difference was also evident in the capacity of N-AcGIP to reduce both osteoclast formation and resorption at concentrations 10 times lower than observed with (D-Ala²)GIP. This likely reflects stronger activation of the GIPr and is important in the light of new development for a drug that would target the GIP/GIPr pathway for the treatment of high bone resorption. Indeed, a recent independent study has shown that slighter interactions with residues of helices 6 and 7 of the GIPr by N-AcGIP, as compared with native GIP, can ultimately improve bioactivity of the peptide [39].

In healthy rats treated with N-AcGIP we previously failed to demonstrate any reduction in the number of osteoclasts in trabecular bone, and we also did not observe a reduction in serum markers of bone resorption [24]. However, in the present study, we evidenced a significant decrease in osteoclast numbers as well as CTx in OVX animals treated with N-AcGIP. Furthermore, (D-Ala²)GIP directly reduced the number of osteoclasts and the extent of resorption in RANKL-stimulated osteoclasts. These observations might seem opposed, but are actually in agreement with previous published observations where GIP blocked resorption of stimulated but not resting osteoclasts [13,14]. Indeed, although GIP did not reduce the extent of resorption of crude osteoclasts cultured on dentine slices, it significantly reduced resorption of PTH-stimulated osteoclasts [15,16]. However, due to the emergent role of osteocytes as a major source of RANKL in bone [40] and the growing body of evidence of osteocytic osteolysis in challenging conditions such as lactation [41], it would be interesting in the future to ascertain whether GIP analogues can affect osteocyte function and potentially osteocytic osteolysis.

The present study revealed a marked difference in the concentration of GIP and its analogues required to induce a direct response on bone cells. Previously, we reported that osteoblasts modified the pattern of collagen deposition and cross-linking at concentrations of (D-Ala²)GIP as low as 10 pM, close to the physiological concentration range of GIP [38]. However, the concentrations required to directly alter osteoclast formation and resorption were up to 1000 times higher. These high concentrations of GIP, also employed by others [15], are supra-physiological and appear contrary to the physiological role of GIP. However, in our assay, osteoclasts were only primed with RANKL, whereas in vivo, osteoclasts would receive a plethora of endocrine and local signals that these cells then integrate to elicit appropriate biological responses. Several hormones released after a meal have anti-resorptive activities and directly inhibit bone resorption, such as GLP-2 and amylin [10,42–44]. It is plausible that the observed reduction in CTx observed after a meal represents the overall integrated response of osteoclasts to all the signals, rather than a direct response to only one mediator. Nevertheless, the elevated GIP concentrations required to affect osteoclasts may also have a strong impact on the strategy to target this pathway for the treatment of osteoporosis. As such, clinically available DPP-4 inhibitor drugs, that hamper the degradation of endogenous gut hormones, would only induce a modest rise in endogenous concentrations of circulating GIP. Thus, the administration of a bolus of exogenous GIP analogue appears a better option to target both osteoblasts and osteoclasts.

In order to advance a new therapeutic option, detailed information is required concerning the mechanism of action. The GIPr is a G protein-coupled receptor that signals intracellularly, in several cell types including osteoblasts, through sustained activation of adenylyl cyclase by the Go subunit resulting in elevation of intracellular cAMP and PKA activation. Surprisingly, the use of several pharmacological inhibitors of adenylyl cyclase, phosphodiesterase or PKA did not inhibit GIP-mediates effects on osteoclast formation, suggesting that in osteoclast precursors, GIP signals independently of this classical pathway. Indeed, the action of (D-Ala²)GIP was mediated by an inhibition of the rise in [Ca²⁺], and a reduction in Ca²⁺ oscillations that lowered calcineurin activity and subsequently sequestered NFATc1 in the cytoplasm. Fig. 7 summarizes the mode of action of GIP. This contrasts with the established actions of GIP on pancreatic beta-cells [45] and further studies are required to elucidate how Ca²⁺ influx is blocked in osteoclasts.

In conclusion, the present study has clearly evidenced that GIP analogues directly reduce osteoclast formation and resorption in a dose-dependent manner by blocking RANKL-mediated [Ca²⁺] oscillations and cytoplasmic sequestration of NFATc1. Pharmacological concentrations required for such effects provide the impetus to design and exploit enzymatically stable GIP analogues for the treatment of bone resorption disorders in humans.

**Fig. 7.** Mechanism of action of GIP. Overall GIP blocks osteoclast precursor differentiation into osteoclast by reducing RANKL-induced [Ca²⁺] thereby inhibiting calcineurin activation and hence NFATc1 translocation. Surprisingly, GIP acts independently of the classical adenylyl cyclase (AC) pathway. But GIP also inhibits bone resorption by mature osteoclasts through a mechanism that as yet to be determined.
Acknowledgements

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References