Nitensidine A, a guanidine alkaloid from *Pterogyne nitens*, induces osteoclastic cell death

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Abstract Nitensidine A is a guanidine alkaloid isolated from *Pterogyne nitens*, a common plant in South America. To gain insight into the biological activity of *P. nitens*-produced compounds, we examined herein their biological effects on osteoclasts, multinucleated giant cells that regulate bone metabolism by resorbing bone. Among four guanidine alkaloids (i.e., galegine, nitensidine A, pterogynidine, and pterogynine), nitensidine A and pterogynine exhibited anti-osteoclastic effects at 10 μM by reducing the number of osteoclasts on the culture plate whereas galegine and pterogynidine did not. The anti-osteoclastic activities of nitensidine A and pterogynine were exerted in a concentration-dependent manner, whereas nitensidine A exhibited an approximate threefold stronger effect than pterogynine (IC_{50} values: nitensidine A, $0.93 \pm 0.024 \mu\text{M}$; pterogynine,

$2.7 \pm 0.40 \mu\text{M}$). In the present study, the anti-osteoclastic effects of two synthetic nitensidine A derivatives (nitensidine AT and AU) were also examined to gain insight into the structural features of nitensidine A that exert an anti-osteoclastic effect. The anti-osteoclastic effect of nitensidine A was greatly reduced by substituting the imino nitrogen atom in nitensidine A with sulfur or oxygen. According to the differences in chemical structures and anti-osteoclastic effects of the four guanidine alkaloids and the two synthetic nitensidine A derivatives, it is suggested that the number, binding site, and polymerization degree of isoprenyl moiety in the guanidine alkaloids and the imino nitrogen atom cooperatively contribute to their anti-osteoclastic effects.

Keywords *Pterogyne nitens* · Guanidine alkaloids · Osteoclast · Cytotoxicity · Structure and activity relationship

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Introduction

Osteoclasts are multinucleated primary bone-resorbing cells that differentiate from hematopoietic stem cells (Edwards and Mundy 2011). Hematopoietic stem cells are differentiated into mononuclear osteoclasts in the presence of cytokines, macrophage-colony stimulating factor (M-CSF) (Felix et al. 1990; Yoshida et al. 1990), and receptor activator of nuclear factor- κB

(NF- κ B) ligand (RANKL) (Yasuda et al. 1998; Lacey et al. 1998), which are produced by osteoblasts. Under the control of these cytokines, mononuclear osteoclasts are multinucleated and actively resorb bone. Although bone resorption is an essential physiological event for bone remodeling and tooth eruption, excessive bone resorption by osteoclasts causes several metabolic bone disorders including osteoporosis, arthritis, periodontitis, bone metastasis, corticosteroid-induced bone loss, and Paget's disease (Rodan and Martin 2000; Chambers 2000; Teitelbaum 2000; Boyle et al. 2003). Therefore, compounds that specifically induce osteoclastic cell death could be developed as anti-osteoclastic drugs for the treatment of the abovementioned metabolic bone disorders.

A native tree *Pterogyne nitens*, common in South America (Bolzani et al. 1995; Regasini et al. 2008; Fernandes et al. 2008; Regasini et al. 2009, 2010; Duarte et al. 2010) has been found to produce flavonoids, such as kaempferol, quercetin, and rutin, which have been reportedly found to exert anti-osteoclastic effects (Wattel et al. 2003; Woo et al. 2004). In addition to the flavonoids, we have also revealed that *Pterogyne nitens* produced cytotoxic guanidine alkaloids (Regasini et al. 2009; Duarte et al. 2010), whose biological activities against osteoclasts have not been reported. In the present study, therefore, we examined the anti-osteoclastic effects of four guanidine alkaloids isolated from *Pterogyne nitens* (i.e., galegine, nitensidine A, pterogynidine, and pterogynine) to gain insight into the biological activity of *Pterogyne nitens*-produced compounds and understand the various possibilities of the plant for ethnomedicinal use. Among the four guanidine alkaloids, the anti-osteoclastic effect of nitensidine A was the most effective. Therefore, the effects of synthesized nitensidine A derivatives were also examined in this study to gain insight into the structural features of nitensidine A that exert an anti-osteoclastic effect.

Materials and methods

Guanidine alkaloids

The guanidine alkaloids tested in the present study were obtained from *Pterogyne nitens* leaves or branches. Briefly, the *Pterogyne nitens* leaves or branches were macerated in ethanol at room temperature and the

extract was separated by gel permeation chromatography, reversed-phase silica gel column chromatography and reversed-phase HPLC according to the procedure previously reported (Regasini et al. 2009, 2010).

Synthesis of nitensidine A derivatives

Synthetic nitensindines AT and AU were prepared as described previously, with minor modifications (Venkatachalam et al. 2003). A solution of 1,1'-carbonyldiimidazole (1.0 mmol) or 1,1'-thiocarbonyldiimidazole (1.0 mmol) in dried *p*-dioxane (15 ml) was added to a solution of geranylamine (2.2 mmol) in dried *p*-dioxane (3.0 ml), at 5 °C. This mixture was stirred for 2 h, at room temperature. The solvent was removed under reduced pressure. The residue was partitioned three times in ethyl acetate and then filtered. The filtrate was partitioned three times with saturated aqueous citric acid solution (two times), saturated aqueous sodium bicarbonate (two times), water (two times), dried over magnesium sulphate. The crude products were purified over a silica gel column using isocratic system of hexane–ethyl acetate (3:2) to afford nitensindines AT and AU. Structures of these compounds were established by ^1H and ^{13}C NMR spectral analysis. Chemical shifts (δ) were expressed in ppm. Coupling constants (J) were expressed in Hz, and splitting patterns are described as follows: s = singlet, br s = broad singlet, t = triplet, m = multiplet.

Nitensidine AT

This yellow oil was obtained in 55 % yield. ^1H NMR (500 MHz, CDCl_3) δ 5.93 (br s; 3-NH, 4-NH), 5.17 (t; J = 7.0 Hz, H-2'), 4.99 (t; J = 7.0 Hz, H-6'), 3.93 (br s; H-1'), 2.01 (m; H-5'), 1.96 (m; H-4'), 1.61 (s; H-9' and H-10'), 1.52 (s; H-8'). ^{13}C NMR (125 MHz, CDCl_3) δ 181.2 (C-2), 140.7 (C-3'), 131.6 (C-7'), 123.4 (C-6'), 118.8 (C-2'), 42.3 (C-1'), 39.2 (C-4'), 26.1 (C-5'), 25.4 (C-9'), 17.4 (C-8'), 16.2 (C-10').

Nitensidine AU

This white solid was obtained in 21 % yield. ^1H NMR (500 MHz, CDCl_3) δ 5.17 (t; J = 6.0 Hz, H-2'), 4.97 (t; J = 6.0 Hz, H-6'), 4.52 (br s; 3-NH, 4-NH), 3.65 (t; J = 6.0 Hz, H-1'), 1.96 (m; H-5'), 1.89 (m; H-4'), 1.58 (s; H-10'), 1.56 (s; H-10'), 1.50 (s; H-8'). ^{13}C NMR (125 MHz, CDCl_3) δ 158.5 (C-2), 139.0 (C-3'), 134.6

(C-7'), 123.8 (C-6'), 121.0 (C-2'), 39.4 (C-1'), 38.4 (C-4'), 26.4 (C-5'), 25.5 (C-9'), 17.5 (C-8'), 16.1 (C-10').

Biochemicals

Leukoprol (recombinant human M-CSF) was purchased from Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan). GST-human sRANKL (GST-RANKL) was purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). Recombinant human TGF- β_1 (TGF- β_1) was purchased from R&D Systems, Inc. (Minneapolis, MN, USA). Minimum essential medium Eagle (α -MEM) and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT reagent) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (4.5 g/l glucose) (DMEM), Antibiotic–Antimycotic Mixed Stock Solution (100 $\times$) (Stabilized), Fast red violet LB salt, naphthol AS-MX phosphate, and 2.5 g/l trypsin-1 mM EDTA were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). All other chemicals used were of analytical grade.

Preparation of mouse osteoclasts from bone marrow cells

For the preparation and culture of mouse osteoclasts, α -MEM supplemented with 10 % (v/v) heat-inactivated fetal calf serum (ICN Biomedicals, Inc., Aurora, OH, USA), 4 mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 250 ng/ml amphotericin B was used in the present study, and the cells were cultured at 37 °C in a humidified atmosphere of 5 % (v/v) CO₂ in air.

Mouse osteoclasts were prepared from mouse bone marrow cells of 6- to 9-week-old male ddY mice (SLC Inc., Shizuoka, Japan). In brief, mouse bone marrow cells ($1.0\text{--}1.5 \times 10^7$ cells/dish/6 ml) were cultured for the first 3 days in the presence of 4,000 U/ml recombinant human M-CSF (M-CSF), 1 ng/ml recombinant human TGF- β_1 (TGF- β_1), and 100 ng/ml GST-human sRANKL (GST-RANKL) in 100-mm diameter type I collagen-coated culture dishes (AGC Techno Glass Co., Ltd., Chiba, Japan), where GST-RANKL and Leukoprol were used for preparation of mouse osteoclasts instead of RANKL and M-CSF, respectively, according to previous reports (Mochizuki et al. 2006; Zhao et al. 2009; Tomimori et al. 2009). TGF- β_1 was added to the culture medium together with M-CSF and GST-RANKL according to previous reports to

promote the efficiency of osteoclast differentiation (Sells Galvin et al. 1999; Kaneda et al. 2000; Fuller et al. 2000; Yan et al. 2001). After 3 days of culture, adherent cells were harvested by treatment with trypsin–EDTA, and the resulting harvested cells (3×10^4 cells/well/100 μ l) were placed on 96-well half area culture plates (Corning Inc., Corning, NY, USA). After culture on the plates for 24 h, the culture medium was replaced with fresh medium that contained 4,000 U/ml M-CSF and 100 ng/ml sRANKL, and the cells were further cultured with 0.5 % (v/v) vehicle [dimethyl sulfoxide (DMSO) (Nacalai Tesque, Inc., (Kyoto, Japan))] or test compounds for 24 h.

The present study with experimental animals was approved by and conducted in accordance with the guidelines of the Animal Experiment Committee of Chubu University.

TRAP staining

Osteoclasts cultured with 0.5 % (v/v) vehicle (DMSO) or test compounds for 24 h were stained for tartrate-resistant acid phosphatase (TRAP), a typical marker enzyme of osteoclasts, as previously described (Nakagawa et al. 2002, 2003, 2007) to visualize osteoclasts. In brief, the cells were fixed with 3.7 % formaldehyde in phosphate buffered saline (PBS)(–) for 15 min. After treatment with ethanol/acetone (1:1) for 1 min, the well surface was dried and treated with the TRAP staining solution [0.1 M sodium acetate buffer (pH 5.0) containing 50 mM sodium tartrate, 0.1 mg/ml naphthol AS-MX phosphate, and 1 mg/ml fast red violet LB salt] for 30 min at room temperature. The resulting visualized osteoclasts were detected under an All-in-One Fluorescence Microscope, the BZ-8100 BIOZERO (KEYENCE Corp., Osaka, Japan), where the number of osteoclasts having more than five nuclei was also counted. Data are expressed as the mean values $\pm$ SD of six replicate cultures in one of three experiments.

MTT assay

The MTT assay was performed to evaluate cellular sensitivity to nitensidine A as previously described (Nakagawa et al. 2009). In brief, the cells (osteoblasts, Caco-2 (generously provided by Dr. Shinji Yamashita (Setsunan University)), KB-3-1 (generously provided by Dr. Kazumitsu Ueda (Kyoto University)), MCF-7

(generously provided by Prof. Hideki Enokida (Kagoshima University)) and HepG2 cells (obtained from the Riken Cell Bank (Tsukuba, Japan)) were seeded at 3×10^4 cells/well/100 μ l into 96-well culture plates and pre-cultured in α -MEM (osteoblasts) or DMEM (Caco-2, HepG2, KB3-1, and MCF-7 cells) supplemented with 10 % (v/v) heat-inactivated fetal calf serum, 4 mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 250 ng/ml amphotericin B for 24 h at 37 °C in a humidified atmosphere of 5 % (v/v) CO₂ in air. The cells were further cultured with nitensidine A for 72 h, treated with 500 μ g/ml MTT reagent for 4 h, and then 10 % (w/v) SDS in PBS(–) (100 μ l/well) was added to solubilize MTT-formazan produced by enzyme within the cell. After overnight incubation, absorbances at 570 and 630 nm were measured as test and reference wavelengths, respectively. Data are expressed as the mean values $\pm$ SD of four replicate cultures in one of three experiments.

Statistical analysis

Statistical analyses were performed using Microsoft Excel 2010 software (Microsoft Co., Redmond, WA, USA). The statistical significance of differences was determined using the Student's *t* test. *P* values <0.01 were considered statistically significant.

Results

To gain insight into the biological activity of *Pterogyne nitens*-produced compounds, we examined the anti-osteoclastic effects of four *Pterogyne nitens*-produced

guanidine alkaloids (i.e., galegine, nitensidine A, pterogynidine, and pterogynine), which are guanidine derivatives that have different numbers of isoprenyl moieties at their amino nitrogens (Fig. 1). To assess the anti-osteoclastic effects of the four alkaloids, osteoclasts were cultured with each of them at 10 μ M for 24 h. As shown in Fig. 2a, galegine, whose amino nitrogen has one isoprenyl moiety, and pterogynidine, whose amino nitrogen has two isoprenyl moieties, appeared to exert no cytotoxic effects on osteoclasts as well as the 0.5 % (v/v) vehicle (DMSO). In contrast, nitensidine A, whose two amino nitrogens have one geranyl moiety, and pterogynine, whose two amino nitrogens have one isoprenyl moiety, appeared to exert cytotoxic effects against osteoclasts because they markedly reduced the number of cells. To elucidate the anti-osteoclastic effects of the four guanidine alkaloids in detail, the number of multinucleated osteoclasts (>5 nuclei) was counted as shown in Fig. 2b. The number of multinucleated cells in the culture wells was not significantly reduced by either 10 μ M galegine or 10 μ M pterogynidine. In contrast, the number of multinucleated cells was significantly reduced by 10 μ M nitensidine A or pterogynine because few multinucleated osteoclasts were observed in the culture wells, suggesting that these guanidine alkaloids induced osteoclastic cell death. Thus, osteoclasts were cultured with different concentrations of nitensidine A or pterogynine for 24 h to elucidate how they reduced the number of multinucleated cells. As shown in Fig. 3, both of the guanidine alkaloids reduced the number of multinucleated osteoclasts in a concentration-dependent manner. Upon comparing the IC₅₀ value of nitensidine A with that of pterogynine, which were calculated from the

Fig. 1 Structures of the guanidine alkaloids

Galegine

Nitensidine A

Pterogynidine

Pterogynine

Fig. 2 Anti-osteoclastic effect of guanidine alkaloids. Mononuclear osteoclasts were placed on 96-well half area culture plates. After culture for 24 h, the resulting multinucleated osteoclasts were cultured with or without 10 μ M guanidine alkaloids for 24 h. After culture, the cells were stained for TRAP (a), and then multinucleated osteoclasts (>5 nuclei) were counted (b). Bar = 200 μ m. Similar results were obtained in at least two other experiments. Data are expressed as the mean values $\pm$ SD of six replicate cultures in one of three experiments. Ctrl control, GAL galegine, NIN nintensidine A, PGD pterogynidine, PGN pterogynine. Asterisks, $P < 0.01$ compared with control

concentration–viability curve shown in Fig. 3, the value of nintensidine A was approximately threefold lower than that of pterogynine (IC_{50} value: nintensidine A, 0.93 ± 0.024 μ M; pterogynine, 2.7 ± 0.40 μ M), indicating that

Fig. 3 Concentration-dependent anti-osteoclastic effects of nintensidine A and pterogynine. Mononuclear osteoclasts were placed on 96-well half area culture plates. After culture for 24 h, they were cultured with nintensidine A (circles) and pterogynine (triangles) at different concentrations for 24 h. After culture, the cells were stained for TRAP, and then multinucleated osteoclasts (>5 nuclei) were counted. Similar results were obtained in at least two other experiments. Data are expressed as the mean values $\pm$ SD of six replicate cultures in one of three experiments. Asterisks, $P < 0.01$ compared with the control

nintensidine A is approximately threefold more effective than pterogynine. In the present study, the cytotoxicities of nintensidine A against osteoblasts and 4 cell lines were also examined to judge whether the cytotoxic effect of nintensidine A is specific to osteoclasts or not. As shown in Table 1, nintensidine A exerted cytotoxicities against osteoblasts and the 4 cell lines at approximately 10- to 40-fold higher concentration than its cytotoxicity against osteoclasts.

In the present study, we also examined the anti-osteoclastic effects of synthetic nintensidine A derivatives (nintensidine AT and AU) to begin structure–activity relationship (SAR) analysis and obtain insight into the structural features of nintensidine A that exert an anti-osteoclastic effect. To this end, the imino nitrogen atom (N) in nintensidine A was substituted with sulfur (S) (nintensidine AT) or oxygen (O) (nintensidine AU) at the beginning of SAR analysis (Fig. 4a). As shown in Fig. 4b, c, more than 75 % of the multinucleated osteoclasts remained in the culture wells when the cells were treated with nintensidine AT or AU at 10 μ M for 24 h, indicating that the substitution of the imino nitrogen atom in nintensidine A with sulfur or oxygen substantially reduced the anti-osteoclastic effect.

Fig. 4 Anti-osteoclastic effects of synthetic nitensidine A derivatives. **a** Structures of nitensidine A and its synthetic derivatives. **b, c** Mononuclear osteoclasts were placed on 96-well half area culture plates. After culture for 24 h, they were cultured with or without 10 μ M nitensidine A or its derivatives for 24 h. After culture, the cells were stained for TRAP, and then multinucleated osteoclasts (>5 nuclei) were counted. Bar = 200 μ m. Similar results were obtained in at least two other experiments. Data are expressed as the mean values $\pm$ SD of six replicate cultures in one of three experiments. Ctrl control, NIN nitensidine A, NIN-AT nitensidine AT, NIN-AU nitensidine AU. Asterisks, $P < 0.01$ compared with nitensidine A

Discussion

In the present study, we examined the anti-osteoclastic effects of guanidine alkaloids isolated from *Pterogyne*

Table 1 Cytotoxicity of nitensidine A against osteoclasts and four cell lines

Cells	IC ₅₀ (μ M)
Osteoclasts	0.93 $\pm$ 0.042
Osteoblasts	19 $\pm$ 0.39
Caco-2 cells	28 $\pm$ 0.63
HepG2 cells	11 $\pm$ 0.57
KB3-1 cells	36 $\pm$ 0.35
MCF-7 cells	14 $\pm$ 0.0

Data are expressed as the mean values $\pm$ SD of four replicate cultures in one of three experiments

nitens, a common plant in South America, and first found that nitensidine A and pterogynine exert anti-osteoclastic effects via reducing the number of multinucleated osteoclasts in culture wells by focusing on the cytotoxicity of guanidine alkaloids against osteoclasts. In contrast, the studies to elucidate the effects of guanidine alkaloids on osteoclastogenesis and expression of the genes regulated by M-CSF and RANKL are currently in progress since osteoclasts differentiate from hematopoietic stem cells in the presence of these cytokines (Felix et al. 1990; Yoshida et al. 1990; Yasuda et al. 1998; Lacey et al. 1998).

Although the mechanism for how nitensidine A and pterogynine reduce the number of osteoclasts is unclear, both apoptosis and necrosis must be involved in the anti-osteoclastic effect as previous reports have described using other cell types (Bolzani et al. 1995; Regasini et al. 2009; Duarte et al. 2010). Instead, four guanidine alkaloids and two nitensidine A derivatives were prepared in the present study and used in preliminary SAR analysis to obtain insight into the structural features of nitensidine A that exert an anti-osteoclastic effect. Based on the relationship between the structures and anti-osteoclastic effects of the four guanidine alkaloids tested (galegine, nitensidine A, pterogynidine, and pterogynine), it is suggested that the number and position of the isoprenyl moiety binding to guanidine could determine the anti-osteoclastic effect. The present study at least indicated that the isoprenyl moiety could confer anti-osteoclastic effects onto guanidine. Based on the relationship between the structures and anti-osteoclastic effects of nitensidine A and pterogynine, it is suggested that the polymerization degree of isoprenyl moiety could enhance the anti-osteoclastic effect of guanidine alkaloid. In addition to the importance of the number,

binding site, and polymerization degree of isoprenyl moiety in the guanidine alkaloids, synthetic nitensidine A derivatives clearly indicated the importance of the imino nitrogen atom in the guanidine core unit. Although extensive SAR analysis with other nitensidine A analogs would be required to fully understand how nitensidine A exerts anti-osteoclastic effects, the generation of imino tautomeric forms of guanidine may play a crucial role in their anti-osteoclastic effects as mentioned in our previous study (Regasini et al. 2009).

Excessive bone resorption by osteoclasts is strongly implicated in the pathogenesis of several bone disorders, such as osteoporosis, arthritis, periodontitis, bone metastasis, corticosteroid-induced bone loss and Paget's disease (Rodan and Martin 2000; Chambers 2000; Teitelbaum 2000; Boyle et al. 2003). Although nitensidine A was the most effective among the six compounds tested, nitensidine A seems to develop side effects in vivo since nitensidine A exerted cytotoxicity against HepG2 cells at about only 10-fold higher concentrations than that against osteoclasts. Nitensidine A seems to have substantial cytotoxic off-target effects in vivo and does not so specifically exert cytotoxicity against osteoclasts among normal cells since the cytotoxicity of nitensidine A against osteoclasts was exerted at about 20-fold lower concentrations than that against osteoblasts as shown in Table 1. However, nitensidine A could be a promising lead compound for the development of an anti-osteoclastic drug for the treatment of above-mentioned metabolic bone disorders.

Collectively, the number, binding site, and polymerization degree of isoprenyl moiety in the guanidine alkaloids and the imino nitrogen atom in the guanidine core unit would cooperatively contribute to the anti-osteoclastic effects of guanidine alkaloids.

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