

REVIEW ARTICLE

Microbial metabolites and derivatives targeted at inflammation and bone diseases therapy: chemistry, biological activity and pharmacology

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Microbial metabolites have attracted increasing interest as a source of therapeutics and as probes for biological mechanisms. New microbial metabolites and derivatives targeted at inflammation and bone disease therapy have been identified by focusing on prostaglandin release, osteoblast differentiation and immune cell functions. These modulators of inflammatory processes and bone disease contribute to our understanding of biological mechanisms and support identification of the therapeutic potential of drug lead candidates. The present review describes recent advances in the chemistry and analysis of inhibitors of prostaglandin release or other functional molecules of immune cells, as well as inducers of osteoblast differentiation, including biological and pharmacological activities.

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INTRODUCTION

Professor Hamao Umezawa directed fundamental studies underpinning the development of antibiotics and their derivatives for use in the treatment of infectious disease and cancer during 1960s–1980s. Considering the importance of inflammatory processes as drug targets, Umezawa and Aoyagi commenced exploratory research to identify low-MW enzyme inhibitors from microbial metabolites. Their discovery of numerous protease inhibitors has permitted analysis of disease mechanisms and therapies for inflammation.¹ Of the proteases involved in inflammatory processes, serine–cysteine proteases, plasmin, chymotrypsin, elastase, prolyl endopeptidase and thiol protease have crucial roles in the coagulation, fibrinolytic and kinin systems. Leupeptin,² chymostatin³ and poststatin⁴ were identified as inhibitors of plasmin, chymotrypsin and prolyl endopeptidase, respectively by, Aoyagi *et al.* In addition, elasnin was discovered as an inhibitor of elastase by Omura *et al.*⁵

Research on inflammation has since expanded to include inflammatory bone disease. Dysregulated inflammation leads to increased bone resorption and suppressed bone formation. Thus, the inhibition of bone resorption is a therapeutic target for which inhibitors have been screened. For example, ICM0201 isolated from *Cunninghamella* sp. F-1490 was discovered as an inhibitor of osteoclastogenesis.^{6,7}

Our recent efforts have directed mechanistic study or drug discovery for inflammation and bone disease, focusing on the relationship between inflammation and bone disease, focusing on the following fields: prostaglandin release inhibitors during inflammation; regulating of immune cells during inflammation; and bone repair during bone disease.

Prostaglandin release inhibitors during inflammation

In addition to playing an important role in maintaining physiological homeostasis, prostaglandins have been implicated in several diseases such as inflammatory disorders, sensory neuron-related disorders and cancer. Prostaglandin release inhibitors have the potential to ameliorate inflammation. Thus, a series of prostaglandin release inhibitors are overviewed. Notably, isolation of pronodine A⁸ and sacchathridine A⁹ (two prostaglandin E₂ (PGE₂) release inhibitors) from microbial metabolites and their biological evaluation are described.

Regulation of immune cells during inflammation

Rheumatoid arthritis,¹⁰ a chronic systemic disease characterized by inflammatory erosive synovitis, elicits marked neovascularization, inflammatory cell infiltration and synovial hyperplasia. The infiltration of cells, predominantly monocytes and macrophages into arthritic joints, is observed. Moreover, vascular endothelial growth factor

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Dedication: We are pleased to dedicate this review article to late Professor Hamao Umezawa for his 103 birth years, 31 years after death and a devoted special issue in 2018. He was a pioneer in the field of antibiotic chemotherapy. He was awarded a number of prizes including Japanese Academy award and Paul Ehrlich prize, and many honors including the Japanese Order of Culture and Légion d'honneur. We are deeply grateful for his achievements still giving a number of study guidance to us.

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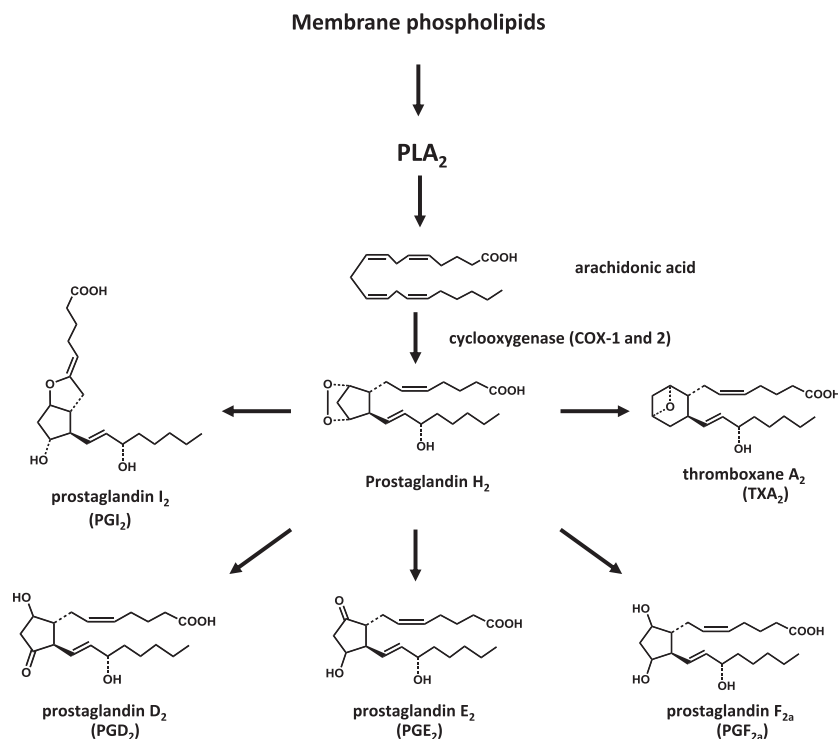


Figure 1 Arachidonic acid cascade.

receptor-1 (VEGFR-1; Flt-1) is functionally expressed in both monocytes and macrophages, which have a crucial role in promoting inflammation.^{11–16} During screening of microbial metabolites to identify novel inhibitors of VEGFR-1 (Flt-1) tyrosine kinase, vegfrecine¹⁷ was isolated from the culture broth of *Streptomyces* sp. MK931-CF8.

Recently, heparanase (endo-β-D-glucuronidase) present within immune cells has been focused on as an inflammatory modulator. Though heparanase is highly relevant for tumor progression and metastasis, the role of heparanase in inflammatory disorders has also attracted much attention in the past decade because a significant increase in the expression and enzymatic activity of heparanase has been reported in numerous inflammatory conditions, typically associated with degradation of heparan sulfate and extensive remodeling of the extracellular matrix.^{18,19} Heparanase inhibitors exhibiting anti-inflammatory effects were overviewed, and inflammatory mechanisms associated with heparanase were studied and elucidated using heparastatin (SF4),²⁰ a synthetic derivative of microbial metabolite siastatin B.²¹

Bone repair during bone disease

Dysregulation of bone loss and subsequent repair is germane to many orthopedic conditions, including inflammatory arthritis, osteoporosis, osteonecrosis, metabolic bone disease, tumors and periprosthetic particle-associated osteolysis. Bone diseases, such as osteoporosis,²² are caused by dysregulated bone remodeling characterized by the sequential tethering of bone formation by osteoblasts and bone resorption by osteoclasts. Understanding the molecular mechanisms for underlying regulation of osteoblast differentiation is of great interest for developing therapeutics for bone disease and bone regenerative medicine. A series of synthetic and natural inducers of osteoblast differentiation have been summarized. Compounds capable of inducing osteoblast differentiation in C3H10T1/2 cells were

screened from libraries of microbial fermentation broths using alkaline phosphatase (ALP) expression. Isolation and biological evaluation of decalpenic acid (DPA),²³ discovered within the fermentation broth of *Penicillium verrucosum* CR37010, have also been described.

This review describes recent progress for studies in investigating the chemistry, biochemistry and pharmacology of pronquodine A, saccharthridine A, DPA, vegfrecine and heparastatin (SF4).

PROSTAGLANDIN RELEASE INHIBITORS DURING INFLAMMATION

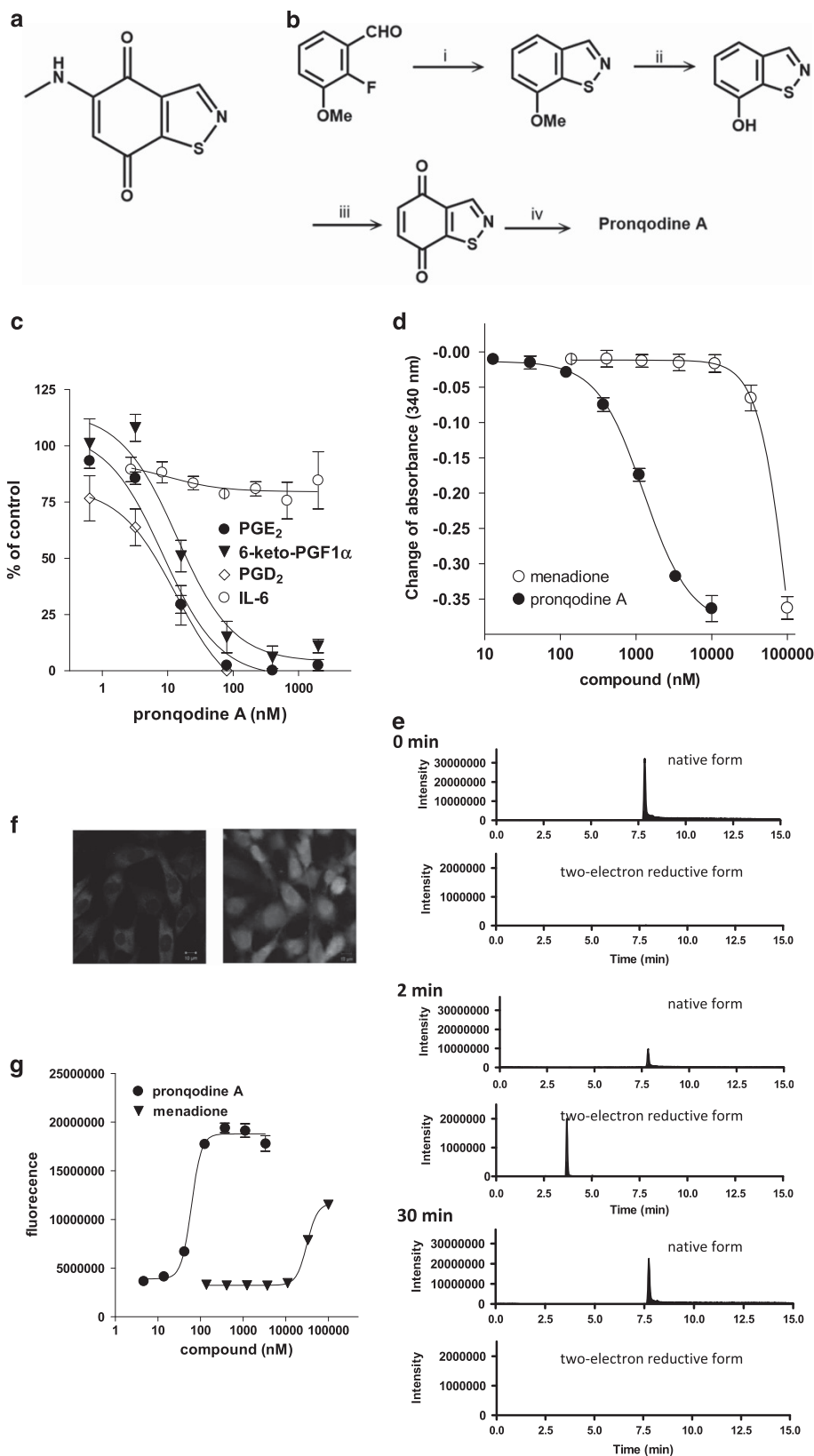
Prostaglandins are bioactive lipid mediators. Arachidonic acid, key for prostaglandin biosynthesis, is enzymatically converted to various kinds of prostaglandins via cyclooxygenase (COX) and prostaglandin synthase (Figure 1). Each prostaglandin binds to its specific G protein-coupled receptor to activate second messengers.²⁴ Prostaglandins have a key role in maintaining physiological homeostasis as well as important roles in several diseases such as inflammatory disorders, sensory neuron-related disorders and cancer.^{25–27}

Although many synthetic inhibitors of prostaglandin release have been developed by targeting inhibition of COX activity, for example, nonsteroidal anti-inflammatory drugs such as indomethacin,²⁸ new compounds with unique mechanisms of action are still needed.²⁹

Natural products are both potential sources of probes for understanding of inflammatory mechanisms and important seed compounds for new anti-inflammatory drugs. Licochalcone A, a major phenolic constituent of the licorice species *Glycyrrhiza inflata* inhibits production of lipopolysaccharide (LPS)-induced PGE₂ by dermal fibroblasts.³⁰ As such, licochalcone A may be beneficial for therapeutic skin care of sensitive or irritated skin. α-Viniferin, an oligostilbene of trimeric resveratrol first isolated from *Caragana chamlagu* has been shown to exert an anti-inflammatory effect via inhibition of prostaglandin H₂.^{31,32} α-Viniferin has also been isolated from *Clematis mandshurica*. α-Viniferin has been shown to decrease LPS-induced

production of nitric oxide and PGE₂, and downregulate LPS-induced expression of proinflammatory genes, such as inducible nitric oxide synthase and COX-2, by suppressing the activity of nuclear factor kappa B via dephosphorylation of Akt/phosphoinositide 3-kinase.³³

Tryptanthrin, isolated from the medicinal plant *Isatis tinctoria* (Brassicaceae) has also been identified as a COX-2 inhibitor.³⁴ Triterpenoids, ursolic acid, 23-hydroxyursolic acid, 3-O- α -L-arabinopyranosyl-23-hydroxyursonic acid and 3-O- β -D-glucopyranosyl-23-



hydroxyursolic acid were isolated from the stem bark of *Cussonia bancoensis* and evaluated for effects on LPS-induced nitric oxide and PGE₂ release using the macrophage cell line RAW 264.7.³⁵ Tectorigenin and tectoridin, isolated from the rhizomes of Korean *Belamcanda chinensis* (Iridaceae) for the treatment of inflammation, suppressed PGE₂ production by rat peritoneal macrophages stimulated by 12-*O*-tetradecanoylphorbol 13-acetate (a protein kinase C (PKC) activator) or thapsigargin, an endomembrane Ca²⁺-ATPase inhibitor.³⁶ Hydroxytyrosol, a major antioxidant constituent of olive oil and cardioprotective polyphenol present in Mediterranean diets, reduced matrix metalloproteinase 9 (MMP-9) and COX-2 induction in activated human monocytes via PKC- α and PKC- β 1 inhibition.³⁷

Inhibitors of prostaglandin release eliciting no effect on *in vitro* COX activity were sought from natural product sources. During primary screening, the inhibitory activity of samples on PGE₂ release induced by bradykinin in human synovial sarcoma SW982 cells was examined. Positive samples were characterized for inhibitory activity on 6-keto-prostaglandin F1 α (a stable prostacyclin (PGI₂) metabolite) production, interleukin-6 release and *in vitro* COX-2 enzyme activity. After screening samples derived from microorganisms and plants, pronqodine A was isolated from the culture medium of *Streptomyces* sp. MK832-95F2.⁸ The structure of pronqodine A was found to comprise a benzo[d]isothiazole-4,7-dione structure using spectral analysis and total synthesis (Figures 2a and b). Pronqodine A inhibited bradykinin-induced prostaglandin release in a concentration-dependent manner, but did not affect the release of interleukin-6 from SW982 cells (Figure 2c). The IC₅₀ values of pronqodine A for PGE₂ production, 6-keto-prostaglandin F1 α production and prostaglandin D₂ production were 9, 17 and 7 nM, respectively.

NAD(P)H dehydrogenase quinone 1 (NQO1) is known to catalyze the conversion of the quinone structure into its two-electron reduced form.³⁸ Figure 2d shows the change of NADH absorbance at 340 nm in an NQO1 activity assay of pronqodine A or menadione. Menadione was converted into its two-electron reductive form menadiol (2-methyl-1,4-naphthalenediol) by NQO1.³⁹ Pronqodine A and menadione decreased the absorbance at 340 nm in a concentration-dependent manner, indicating that both compounds act as electron acceptors (Figure 2d). To identify whether pronqodine A is reduced by NQO1, a liquid chromatography coupled to high-resolution ESIMS analysis was performed on pronqodine A. The two-electron reductive form of pronqodine A converted by NQO1 was identified at 2 min (Figure 2e). This peak was diminished at 30 min, while the peak of pronqodine A itself returned, suggesting that the hydroquinone form of pronqodine A was oxidized back to the quinone type. As a result of the instability of the two-electron reductive form, menadiol formed from menadione by NQO1 is known to undergo reformation to menadione with concomitant formation of reactive oxygen species (ROS) in human embryonic kidney 293 cells expressing NQO1.³⁹ Similarly, the reductive form of pronqodine A was identified to be unstable and immediately autoxidized to its native form. Pronqodine

A caused intracellular ROS production in SW982 cells (Figure 2f), and induced ROS production at a lower concentration than menadione (Figure 2g).

COX and prostaglandin synthases are well documented for their ability to convert arachidonic acid into prostaglandins such as PGE₂, PGI₂ and prostaglandin D₂. Arachidonic acid induced the release of PGE₂, which was inhibited by pronqodine A in a concentration-dependent manner (Figure 3a). Subsequently, the effect of pronqodine A on COX-1 and COX-2 expression was investigated. In SW982 cells, COX-1 expression was undetectable, while pronqodine A did not affect COX-2 expression (Figure 3b). Next, the effect of pronqodine A on COX-2 activity was examined. While treatment with pronqodine A alone did not affect COX-2 activity (Figure 3c), pronqodine A inhibited COX-2 enzyme activity in the presence of NQO1 (Figure 3d).

Taken together, NQO1 converted pronqodine A to a reduced form, which undergoes autoxidation to revert to its native form in a ROS-generating process. Thus, pronqodine A could regulate prostaglandin release by a ROS-dependent mechanism (Figure 3e).

Sacchathridin A isolated from the fermentation broth of strain *Saccharothrix* sp. MI559-46F5 was identified to be a PGE₂ release inhibitor.⁹ The structure of sacchathridin A was found to be a new naphthoquinone derivative with an acetylhydrazino moiety (Figure 4a). Upon examining the effect of sacchathridin A on PGE₂ release from SW982 cells, inhibition was found to occur in a concentration-dependent manner with an IC₅₀ value of 1.0 μ M without affecting cell growth (Figures 4b and c). Thus, sacchathridin A could be a chemical tool for mechanistic analysis of PGE₂ release.

REGULATION OF IMMUNE CELLS DURING INFLAMMATION

Monocytes and macrophages express VEGFR-1 (Flt-1), which has a crucial role in promoting inflammation.^{11–16} Moreover, VEGFR-1 tyrosine kinase signaling modulates the proliferation of bone marrow hematopoietic cells, as well as the immunity of monocytes/macrophages and chronic inflammation.⁴⁰ As such, suppression of VEGFR-1 signaling by a small molecule may significantly inhibit the underlying cause of rheumatoid arthritis.^{40,41}

Vegfrecine was isolated from the culture broth of *Streptomyces* sp. MK931-CF8 during the target-based screening of microbial metabolites for compounds with inhibitory activity against VEGFR-1 (Flt-1) tyrosine kinase. The structure of vegfrecine was determined by NMR and MS analysis combined with synthesis (Figures 5a and b).¹⁷

Vegfrecine exhibited potent inhibitory activity against VEGFR-1 (Flt-1) and other VEGF receptors (VEGFR-2 and -3; Table 1) and also weakly inhibited platelet-derived growth factor receptor- α and - β , fibroblast growth factor and epidermal growth factor tyrosine kinases *in vitro*. VEGFR-1 (Flt-1) is involved in promoting inflammation, while both VEGFR-1 (Flt-1) and VEGFR-2 (kinase insert domain receptor) are responsible for endothelial cell proliferation and blood vessel permeability. VEGFR-3 (Flt-4) participates in lymphatic vessel development. These receptor tyrosine kinases are also essential for tumor angiogenesis and lymphangiogenesis.^{42–44}

Figure 2 (a) Structure of pronqodine A. (b) Synthetic scheme of pronqodine A: (i) S, NH₄OH, 2-MeOCH₂OH; (ii) Py-HCl, 168 °C; (iii) PIFA, EtOH; (iv) MeNH₂, CeCl₃, EtOH. (c) Effect of pronqodine A on 1 nM bradykinin-induced release of prostaglandin E₂ (PGE₂), 6-keto-prostaglandin F1 α and prostaglandin D₂ (PGD₂) and interleukin-6 (IL-6) from SW982 cells. (d) Effect of pronqodine A and menadione as an electron acceptor in the NAD(P)H quinone dehydrogenase 1 (NQO1) activity assay. (e) Positive liquid chromatography coupled to high-resolution ESIMS spectra for pronqodine A and NQO1 reaction mixtures at *m/z* 195.0202–195.0242 (native form of pronqodine A) and 197.0359–197.0399 (two-electron reductive form of pronqodine A) at 0 min (upper), 2 min (middle) and 30 min (lower). Reaction mixtures contained 25 mM Tris-HCl (pH 7.6), 1 mM NADH, 3 mM FAD and 140 μ g ml⁻¹ NQO1, and 180 μ M pronqodine A at 37 °C. (f) Confocal images of reactive oxygen species (ROS) production induced by pronqodine A in SW982 cells. (g) Quantitation of ROS production induced by pronqodine A and menadione in SW982 cells. (c–g) Reprinted with permission from ref. 8. Copyright (2017) American Chemical Society. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

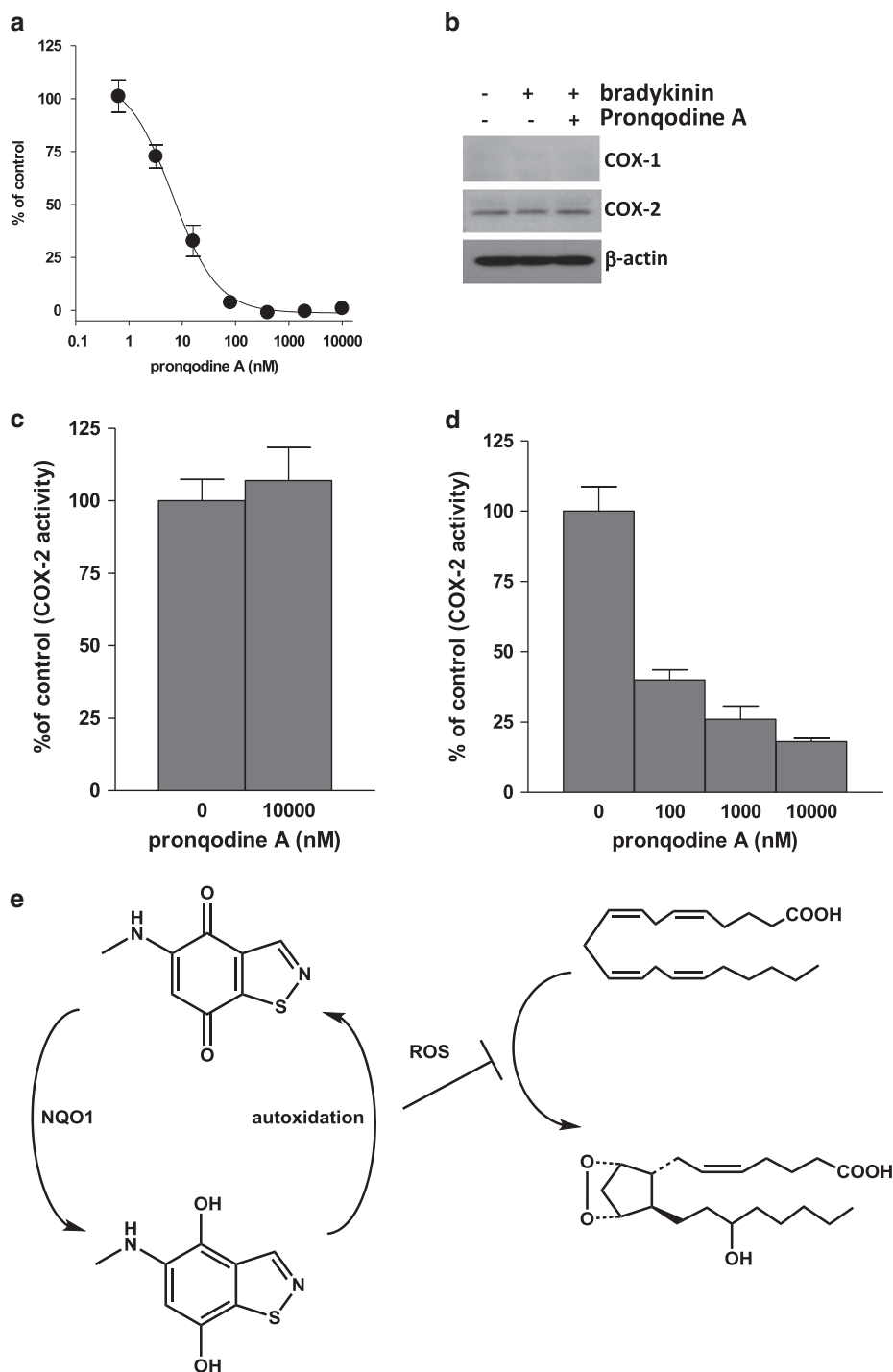


Figure 3 (a) Effect of pronqodine A on $10 \mu\text{g ml}^{-1}$ arachidonic acid-induced prostaglandin E_2 release from SW982 cells. (b) Effect of pronqodine A on expression of cyclooxygenase 1 and 2 (COX-1 and COX-2) in SW982 cells. The concentration of bradykinin was 1 nM for 30 min. (c) Effect of pronqodine A on COX-2 enzyme activity. (d) Effect of pronqodine A with NAD(P)H quinone dehydrogenase 1 (NQO1) on COX-2 enzyme activity. (e) Generation of reactive oxygen species through redox of pronqodine A. (a–d) Reprinted with permission from ref. 8. Copyright (2017) American Chemical Society.

Vegfrecine exhibited a weak inhibitory activity at concentration over $10 \mu\text{M}$ against ligand-induced phosphorylation of VEGFR-1 (Flt-1) in NIH3T3-Flt-1 cells. The inhibitory activity of vegfrecine in cells was 100-fold less than that *in vitro*. Notably, as vegfrecine showed poor solubility in most media, weak inhibitory activity in cells could arise from poor membrane permeability. Thus, improvement of its structural profile would be required.

Heparanase was originally characterized as a matrix degradation enzyme involved in basement membrane degradation and cancer cell invasion. Elevated expression of heparanase was linked to the malignancy and invasiveness of melanoma cells.⁴⁵ Heparanase cleaves the glycosaminoglycan heparan sulfate side chain of heparan sulfate proteoglycans at the glycoside bond of glucuronic acid (Figure 6a). The involvement of heparanase in immune reactions was

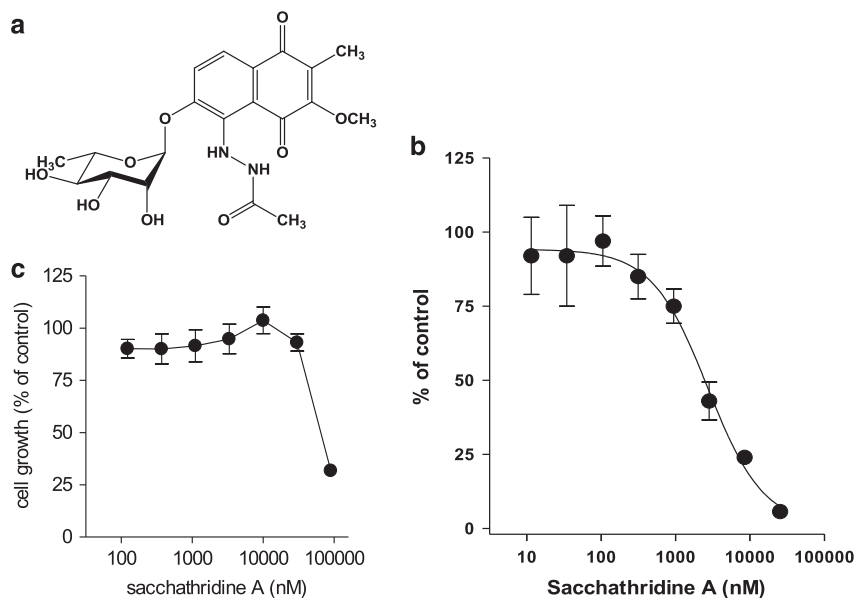


Figure 4 (a) Structure of sacchathridine A. (b) Effect of sacchathridine A on prostaglandin E_2 release from SW982 cells. (c) Effect of sacchathridine A on cell growth of SW982 cells.

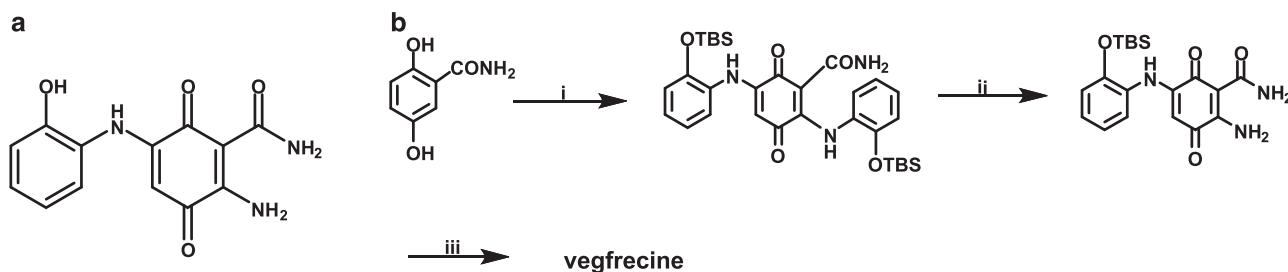


Figure 5 (a) Structure of vegfrecine. (b) Synthetic scheme of vegfrecine: (i) 2-(*t*-butyl-dimethylsilyloxy)aniline, NaIO_4 , MeOH-water, rt, 70%; (ii) 2 N NH_3 , CHCl_3 -MeOH, rt, 95%; (iii) Bu_4NF , AcOH, THF, rt, 90%.

Table 1 Inhibitory activities of vegfrecine and SU5416

	IC_{50} (μM)						
	VEGFR-1 (<i>Flt-1</i>)	VEGFR-2 (<i>KDR</i>)	VEGFR-3 (<i>Flt-4</i>)	PDGF- α	PDGF- β	EGF	FGF
Vegfrecine	0.018	0.011	0.0018	3.2	0.48	2.6	0.73
SU5416	3	1	0.5	0.2	0.4	>100	5

Abbreviations: EGF, epidermal growth factor; FGF, fibroblast growth factor; KDR, kinase insert domain receptor; PDGF, platelet-derived growth factor; VEGFR, vascular endothelial growth factor receptor.

demonstrated by heparan sulfate-degrading activity in immune cells (neutrophils, macrophages and activated T lymphocytes) that resulted in immune cell diapedesis and accumulation within organs.^{46–52} A significant increase in the expression and enzymatic activity of heparanase appears in many inflammatory conditions, associated with degradation of heparan sulfate and remodeling of extracellular matrix.^{19,53}

Heparanase inhibitors, such as heparin and heparin-mimicking compounds, exhibit anti-inflammatory effects both *in vitro* and *in vivo*. Heparin suppressed the development of experimental autoimmune encephalomyelitis (EAE).⁵⁰ PI-88, also known as muparfostat, was examined for activity on eosinophil infiltration in ovalbumin-induced lung allergic inflammation.⁵⁴ PI-88 reduced the number of infiltrating eosinophils. Moreover, mice with

genetic elimination of heparanase exhibited reduced migration of dendritic cells^{55–57} and monocytes.⁵⁸ Sulfated polysaccharide reduced EAE disease severity.^{59,60} The inhibitory activity of chemically modified heparins, such as periodate-oxidized heparin, *N*-desulfated heparin and *N*-desulfated acetylated heparin, appear to correlate with EAE suppression.⁶¹ Effects of the heparanase enzymatic inhibitor ST1514 (glycol split, nonanticoagulant heparin) on delayed-type hypersensitivity reactions were evaluated in the ears of mice. Treatment with ST1514 resulted in decreased ear swelling and edema formation, supporting to the involvement of heparanase in delayed-type hypersensitivity.⁶² Enoxaparin, an analog of the syndecan-1 heparan sulfate chain, reduced the lethality of syndecan-1-deficient mice arising from dextran-induced colitis.⁶³

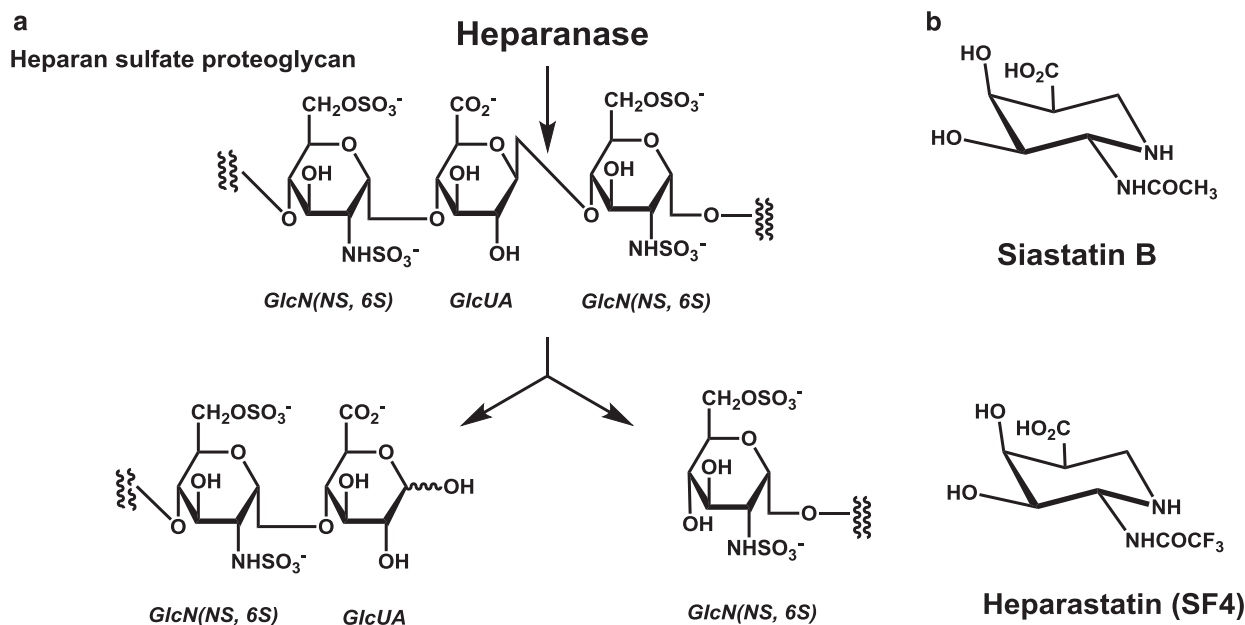


Figure 6 (a) Heparanase cleavage site within the structure of heparan sulfate. (b) Siastatin B and heparastatin (SF4).

Thus far, heparastatin (SF4) has been developed as a heparanase inhibitor for cancer therapy,^{64–67} starting from siastatin B isolated as an inhibitor of sialidase (neuraminidase) from *Streptomyces* culture by Umezawa and Aoyagi (Figure 6b).²¹ Heparastatin (SF4) exhibits potent inhibitory activity ($IC_{50} = 1.02 \pm 0.29 \mu\text{M}$) against recombinant human heparanase (endo- β -glucuronidase) in human melanoma A375M cells transfected with pBK-CMV expression vectors containing heparanase cDNA.^{64,68} Heparastatin (SF4) inhibited the invasion of B16BL6 and Lewis lung carcinoma (3LL) cells through reconstituted Matrigel basement membrane.⁶³ Pulmonary colonization, after i.v. transplantation of B16BL6 cells was suppressed by heparastatin (SF4) in a dose-dependent manner.^{66,67} Heparastatin (SF4) also inhibited spontaneous lung metastasis of 3LL cells.⁶⁵

Considering the similarities between invasion of metastatic cancer cells and extravasation of leukocytes, application of heparanase inhibitors to suppression of inflammatory reactions is potentially significant. Recently, heparastatin (SF4) was applied to a dorsal air pouch inflammation model to demonstrate suppression of extravasation of neutrophils and monocytes by the impairment of basement membrane degradation.²⁰ Heparastatin (SF4) suppressed local inflammation in both *N*-formyl-Met-Leu-Phe (fMLP) and carrageenan-induced dorsal air pouch inflammation models. Anti-inflammatory effects of heparastatin (SF4) co-administrated into the air pouch were also evaluated. Numbers of total infiltrated cells in mice treated with heparastatin (SF4) were significantly lower in fMLP-treated (67%) and, in carrageenan-treated (51%) mice compared with mice injected with proinflammatory agents alone (Figure 7a). Numbers of both neutrophils and monocytes were markedly lower in heparastatin (SF4)-injected mice. Ratios of neutrophils/monocytes were similar in the presence of heparastatin (SF4) (Figures 7b and c). However, numbers of infiltrating neutrophils were unchanged in heparastatin (SF4)-treated mice compared with control mice, suggesting heparanase was not involved in neutrophil infiltration in zymosan-induced peritonitis model (Figure 7d).

Concentrations of inflammatory chemokines (KC, MIP-2 and MCP-1) in dorsal exudates collected from the air pouch model were determined. For carrageenan-mediated inflammation, there was no

significant difference in tested concentrations of KC and MIP-2, while MCP-1 was significantly lower in heparastatin (SF4)-injected mice (Figures 7e–g).

Intervention by heparanase in neutrophil migration was investigated using *in vitro* migration and invasion tests. Migratory capacity was measured as the number of neutrophils that passed through an uncoated membrane, whereas invasion capacity was measured as the number of neutrophils that penetrated through a membrane coated with Matrigel. Addition of heparastatin (SF4) into the medium significantly lowered the number of invading neutrophils, but did not alter the number of migrating neutrophils (Figure 8). Taken together, heparastatin (SF4) significantly suppressed infiltrating monocytes and neutrophils in fMLP- or carrageenan-injected air pouches. Also, heparastatin (SF4) suppressed neutrophil invasion *in vitro*. Thus, this study suggested an involvement of heparanase infiltration in mouse inflammatory leukocytes, indicating a therapeutic application of heparastatin (SF4) in the suppression of inflammatory diseases.

BONE REPAIR DURING BONE DISEASE

Bone diseases such as inflammatory arthritis, osteoporosis and osteonecrosis are germane to the reconstitution of lost bone where processes of acute and chronic inflammation have integral roles. Dysregulated inflammation within bone causes increased bone resorption and suppressed bone formation. Crosstalk between inflammatory cells and cells related to bone healing is essential to the formation, repair and remodeling of bone.⁶⁹ Cells related to bone healing consists of mesenchymal stem cells, as well as osteoblast and vascular lineages. Differentiation of mesenchymal stem cells into osteoblasts is an important process for developing bone disease therapies.

The synthetic small-molecule agonist of hedgehog signaling, purmorphamine induced ALP activity as indicated by ALP staining.^{70–73} Natural products exhibiting osteogenic-inducing activity have recently gained much attention in addition to synthetic small molecules. Genistein, an isoflavone, has been shown to increase bone formation and decrease bone absorption.⁷⁴ In addition, structurally related daidzein and formononetin were shown to induce mRNA and protein expression of BMP-2 in the human osteogenetic cell line MG63.⁷⁵

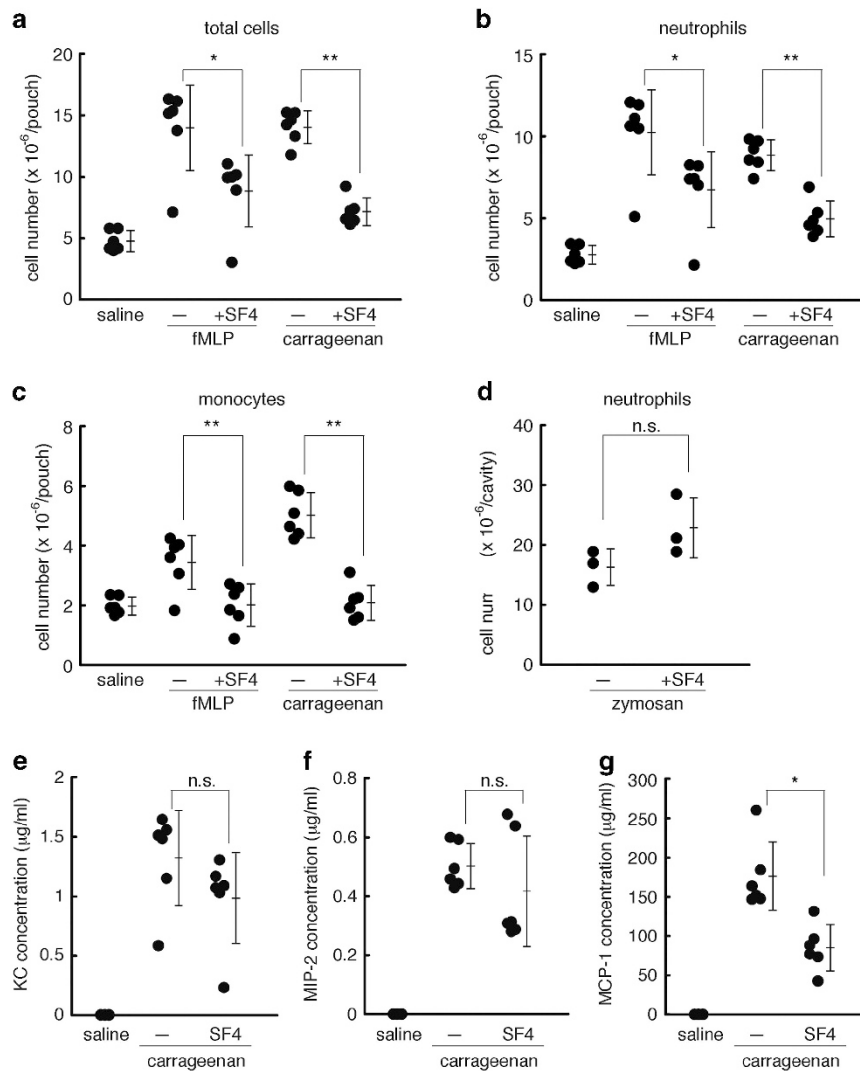


Figure 7 Heparastatin (SF4) suppresses infiltration of neutrophils and monocytes into inflamed air pouches *in vivo*. (a–c) *N*-formyl-Met-Leu-Phe (fMLP, 1 μ M) or carrageenan (1%) in saline with or without 0.1 mM heparastatin (SF4) was injected into air pouches. After 4 h, exudates were collected and numbers of total infiltrating cells (a), neutrophils (b) and monocytes (c) were counted and plotted ($n=6$ per group). (d) Peritoneal exudates derived from zymosan-injected mice were collected. Numbers of neutrophils in the peritoneal fluid were counted ($n=3$). * $P<0.05$, ** $P<0.01$. (e–g) Chemokine concentration in dorsal air pouch exudates. Concentrations of chemokines KC, MIP-2 and MCP-1 in exudates of air pouches injected with carrageenan were measured and plotted ($n=6$ per group). * $P<0.05$. (a–g) Reprinted from ref. 20. Copyright (2017) with permission from Elsevier.

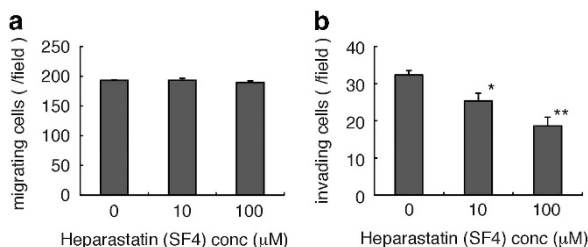


Figure 8 Effect of heparastatin (SF4) on migration and invasion of neutrophils through polycarbonate membranes. Indicated concentrations of heparastatin (SF4) were added to both upper and lower chambers. Numbers of migrating (a) or invading (b) cells on the lower surface were counted after 0.5 or 24 h of incubation, respectively. Data are shown as average \pm s.e. $N=3$, * $P<0.05$, ** $P<0.01$. Reprinted from ref. 20. Copyright (2017) with permission from Elsevier.

Epigallocatechin-3-gallate, a green tea catechin, has a stimulatory effect on human bone marrow stem cells developing toward the osteogenic lineage as evidenced by increased ALP activity, upregulated expression of osteogenic genes and the formation of bone-like nodules.⁷⁶ Honokiol, a major active ingredient of Magnolia extract, is a potent inducer of *in vitro* osteoblast differentiation by virtue of its capacity to suppress basal and tumor necrosis factor alpha-induced nuclear factor kappa B activation, which alleviates the suppressive action of tumor necrosis factor alpha on BMP-2 induced Smad activation.⁷⁷

The mouse mesenchymal cell line C3H10T1/2 expresses mesenchymal stem cell-like pluripotency markers, thus providing a valuable model system. DPA was identified and isolated from the fermentation broth of *P. verruculosum* CR37010 during screening for inducers of osteoblast differentiation of pluripotent mesenchymal C3H10T1/2 cells using an early osteoblast marker (ALP activity).²³ The structure

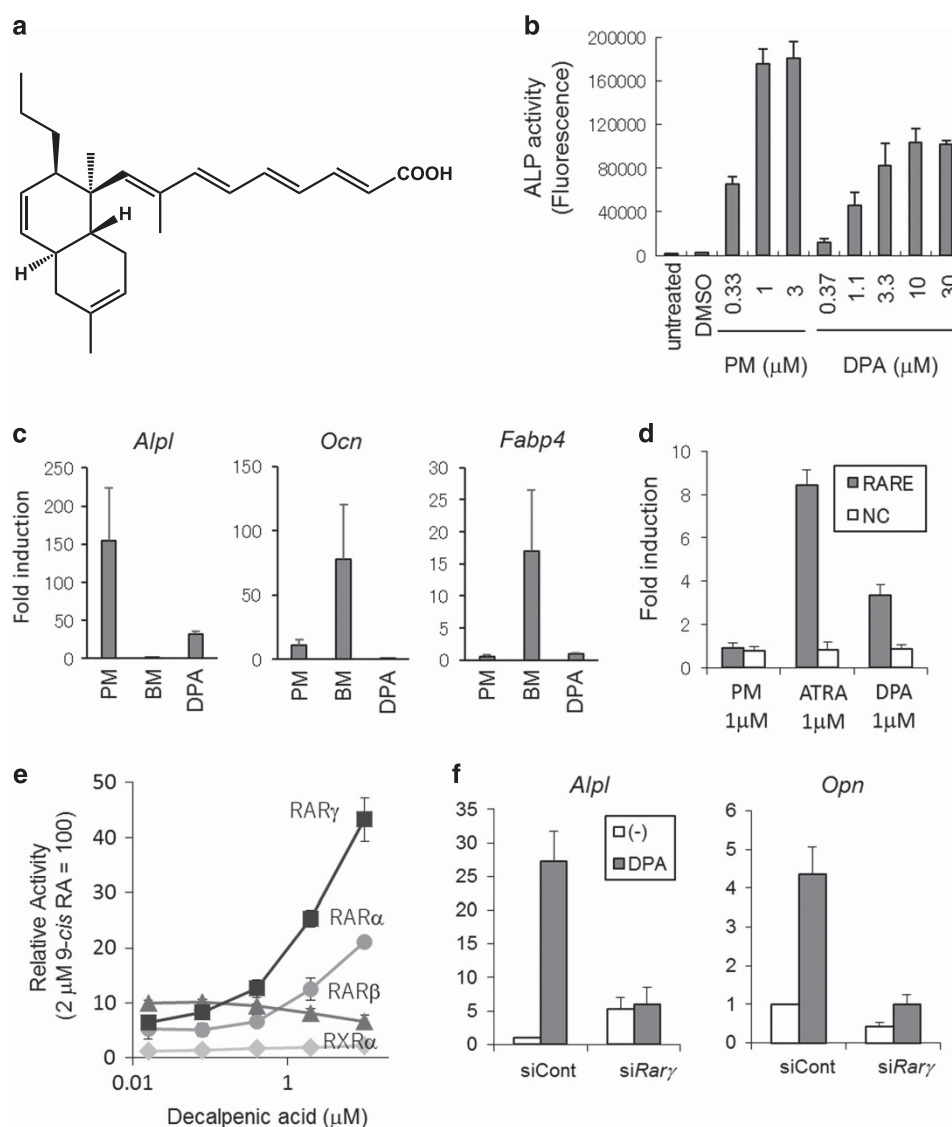


Figure 9 (a) Relative stereochemistry of decalpenic acid (DPA). (b) Induction of an early osteoblastic marker (alkaline phosphatase; ALP) activity by DPA. C3H10T1/2 cells were treated with 0.1% dimethylsulfoxide (DMSO), purmorphamine (PM) or DPA. After 4 days of treatment, ALP activities were measured as described in methods. Data are shown as mean \pm s.d. of three independent experiments performed in duplicate. (c) Real-time PCR analysis of osteoblastic marker genes in C3H10T1/2 cells treated with DPA. C3H10T1/2 cells were treated with 1 μ M PM, 400 ng ml⁻¹ BMP-2 (BM) or 1 μ M DPA. After 8 days of treatment, total RNA was isolated from cells and mRNA expression was determined by real-time PCR analysis for *Alpl*, osteocalcin (*Ocn*) and *Fabp4*. Data were normalized to the level of *Gapdh* and are presented as fold-induction versus untreated controls. Data represent the mean \pm s.d. of three independent experiments. (b, c) Reproduced from ref. 23 with permission. (d) Retinoic acid response element (RARE) reporter assay. Cells were transiently transfected using a Signal RARE reporter kit (Qiagen, Hilden, Germany). After treatment for 24 h with all-*trans* retinoic acid (ATRA), DPA or PM; a hedgehog signaling agonist), the dual luciferase assay was performed. RARE-luc is a reporter plasmid in which the firefly luciferase gene is under the control of tandem RAREs. NC-luc is a negative control reporter plasmid lacking the RARE element. Each error bars represents mean \pm s.d. obtained from three independent samples. (e) Retinoic acid receptor activation profiles of DPA. (f) Effect of retinoic acid receptor gamma knockdown on the biological activity of DPA in C3H10T1/2 cells. Twenty-four hours after *Rar*_{gamma} siRNA transfection, cells were treated with 1 μ M DPA for 48 h, and mRNA levels of *Alpl* and *Opn* were analyzed by real-time reverse transcription-PCR. Each error bar represents the mean \pm s.d. obtained from three independent samples. (d-f) Reproduced from ref. 84 with permission Elsevier. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

of DPA was clarified to possess a tetraenoic acid and propyl side chain on the decalin ring (Figure 9a).

Effects of DPA on osteoblast differentiation of C3H10T1/2 cells were examined. Four-day treatment of cells with 1 μ M purmorphamine induced ALP activity, as measured by ALP staining (Figure 9b). Treatment with DPA also induced ALP activity in a dose-dependent manner. The half maximal effective concentration for DPA based on ALP expression was estimated at 2 μ M. Thus, DPA alone was able to induce ALP activity in C3H10T1/2 cells.

To examine whether DPA could induce the expression of other osteoblast differentiation markers, real-time PCR analysis of a panel of osteoblast marker genes was carried out. Treatment of cells with 1 μ M DPA for 8 days significantly increased mRNA expression of *Alpl*, the gene encoding ALP but did not increase expression levels of the late osteoblast marker *Ocn* (Figure 9c).

C3H10T1/2 cells, like mesenchymal stem cells, can differentiate into various cell types such as adipocytes and osteoblasts, moreover, treatment of C3H10T1/2 cells with BMP-2 induced osteoblastgenesis

and adipocytogenesis.^{78,79} Thus, the effects of DPA on adipogenesis were examined in C3H10T1/2 cells. Treatment with BMP-2 induced adipocyte differentiation, as indicated by *Fabp4* mRNA expression (Figure 9c). In contrast, treatment with 1 μ M DPA or 1 μ M purmorphamine did not induce this adipocyte differentiation marker. These results suggest that treatment with DPA alone did not induce adipocyte differentiation in C3H10T1/2 cells, instead inducing early osteoblastic markers through activation of signaling pathways other than Hedgehog and BMP.

Osteoblast differentiation is stimulated by retinoids.^{80–83} Effects of DPA in C3H10T1/2 cells were compared with all-*trans* retinoic acid, the most important endogenous retinoid.⁸⁴ A reporter assay incorporating a luciferase reporter driven by a multimerized retinoic acid response element demonstrated that DPA induced an approximately fourfold increase in luciferase activity compared with controls (Figure 9d). These data suggest that DPA acts as a retinoid to activate the retinoic acid receptor (RAR) signaling pathway in C3H10T1/2 cells.

Two families of RARs activate transcription by transducing retinoic acid signals: RARs and rexinoid receptors (RXRs).^{85,86} As our results demonstrated that DPA primarily acts as a retinoid, activation of the RARs, RAR α , RAR β and RAR γ , as well as the RXR, RXR α , by DPA was examined. Human embryonic kidney 293T cells transiently transfected with GAL4-fusion constructs for each of the human receptors were investigated for effects of DPA, all-*trans* retinoic acid and 9-*cis* retinoic acid on transcriptional activation at the GAL4 upstream activating sequence (Figure 9e). As previously reported, 9-*cis* retinoic acid showed high efficiency in activating RARs and RXR α .⁸⁴ All-*trans* retinoic acid also showed high activity against RARs and weaker activity against RXR α . In this system, DPA induced significant activation of RAR γ and weak activation of RAR α , whereas DPA caused no significant activation of RAR β and RXR α . These results indicated that DPA is a new retinoid that exhibits selective activity toward RAR β and RXR α .

We examined whether DPA induces early osteoblastic markers (*Alpl* and *Opn*) by activating of RARs. Early osteoblastic markers induced by DPA were inhibited in cells transfected RAR γ -targeting siRNA (Figure 9f). Thus, DPA may be used to specifically activate RAR γ and modulate RAR γ -related biological events.

CLOSING REMARKS

This article describes current progress in the chemical, biochemical and therapeutic potential of microbial metabolites and derivatives targeting inflammation and bone disease. Natural inhibitors affecting target enzymes or receptors associated with inflammation and bone disease have been isolated from microbial metabolites. These inhibitors and a synthetic derivative of a microbial metabolite were used for analysis of inflammatory processes. Pronqodine A inhibits release of prostaglandins (PGE₂, 6-keto-prostaglandin F1 α and prostaglandin D₂) by interrupting the conversion of arachidonic acid to prostaglandins. NQO1 converts pronqodine A to a reduced form that undergoes autoxidation to revert to its native form, generating ROS. This ROS interrupts the process of conversion of arachidonic acid to prostaglandin H₂. Sacchathridine A also inhibited PGE₂ release without affecting cell growth. DPA induces early osteoblastic markers such as ALP activity and *Opn* mRNA in C3H10T1/2 cells via activation of RARs, especially RAR γ . Thus, DPA could be used as a chemical tool for modulating biological events mediated by RAR γ . Vegfrecine was discovered during the target-based screening against VEGFR-1 tyrosine kinase and exhibited selective inhibition against VEGFRs. Heparastatin (SF4) significantly suppressed infiltration of monocytes

and neutrophils in an *in vivo* dorsal air pouch inflammation model, suggesting that cell infiltration can be pharmacologically regulated by heparastatin (SF4). It is reported that heparanase is not always involved in transmigration of immune cells, or that the involvement of heparanase is limited to cell type. Future research will focus on the involvement of cell type-dependent actions of heparanase in inflammatory processes.

Microbial metabolites have been a rich source of drug seeds for inflammation and bone disease and have thus become the special focus of research attention.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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- 1 Aoyagi, T. [An outline of enzyme inhibitors]. *Tanpakushitsu Kakusan Koso* **38**, 1891–1918 (1993).
- 2 Aoyagi, T., Takeuchi, T., Matsuzaki, A., Kawamura, K. & Kondo, S. Leupeptins, new protease inhibitors from Actinomycetes. *J. Antibiot. (Tokyo)* **22**, 283–286 (1969).
- 3 Umezawa, H., Aoyagi, T., Morishima, H., Kunimoto, S. & Matsuzaki, M. Chymostatin, a new chymotrypsin inhibitor produced by actinomycetes. *J. Antibiot. (Tokyo)* **23**, 425–427 (1970).
- 4 Aoyagi, T. *et al.* Poststatin, a new inhibitor of prolyl endopeptidase, produced by *Streptomyces viridochromogenes* MH534-30F3. I. Taxonomy, production, isolation, physico-chemical properties and biological activities. *J. Antibiot. (Tokyo)* **44**, 949–955 (1991).
- 5 Omura, S., Ohno, H., Saheki, T., Yoshida, M. & Nakagawa, A. Elasinin a new human granulocyte elastase inhibitor produced by a strain of *Streptomyces*. *Biochem. Biophys. Res. Commun.* **83**, 704–709 (1978).
- 6 Inoue, H. *et al.* ICM0201, a new inhibitor of osteoclastogenesis from *Cunninghamella* sp. F-1490. I. Taxonomy, fermentation, isolation and biological activities. *J. Antibiot. (Tokyo)* **56**, 209–213 (2003).
- 7 Someno, T., Inoue, H., Kumagai, H., Ishizuka, M. & Takeuchi, T. ICM0201, a new inhibitor of osteoclastogenesis from *Cunninghamella* sp. F-1490. II. Structure determination and synthesis. *J. Antibiot. (Tokyo)* **56**, 214–218 (2003).
- 8 Nakae, K. *et al.* NAD(P)H quinone oxidoreductase 1 (NQO1)-bioactivated pronqodine A regulates prostaglandin release from human synovial sarcoma cells. *J. Nat. Prod.* **76**, 510–515 (2013).
- 9 Nakae, K. *et al.* Sacchathridine A, a prostaglandin release inhibitor from *Saccharothrix* sp. *J. Nat. Prod.* **76**, 720–722 (2013).
- 10 Clague, R. B., Shaw, M. J. & Holt, P. J. Incidence of serum antibodies to native type I and type II collagens in patients with inflammatory arthritis. *Ann. Rheum. Dis.* **39**, 201–206 (1980).
- 11 Adini, A., Kornaga, T., Firozbakht, F. & Benjamin, L. E. Placental growth factor is a survival factor for tumor endothelial cells and macrophages. *Cancer Res.* **62**, 2749–2752 (2002).
- 12 Hattori, K. *et al.* Placental growth factor reconstitutes hematopoiesis by recruiting VEGFR1(+) stem cells from bone-marrow microenvironment. *Nat. Med.* **8**, 841–849 (2002).
- 13 Sawano, A. *et al.* Flt-1, vascular endothelial growth factor receptor 1, is a novel cell surface marker for the lineage of monocyte-macrophages in humans. *Blood* **97**, 785–791 (2001).
- 14 Luttun, A. *et al.* Revascularization of ischemic tissues by PIGF treatment, and inhibition of tumor angiogenesis, arthritis and atherosclerosis by anti-Flt1. *Nat. Med.* **8**, 831–840 (2002).
- 15 Lyden, D. *et al.* Impaired recruitment of bone-marrow-derived endothelial and hematopoietic precursor cells blocks tumor angiogenesis and growth. *Nat. Med.* **7**, 1194–1201 (2001).
- 16 Hiratsuka, S., Minowa, O., Kuno, J., Noda, T. & Shibuya, M. Flt-1 lacking the tyrosine kinase domain is sufficient for normal development and angiogenesis in mice. *Proc. Natl Acad. Sci. USA* **95**, 9349–9354 (1998).
- 17 Nosaka, C. *et al.* Vegfrecine, an inhibitor of VEGF receptor tyrosine kinases isolated from the culture broth of *Streptomyces* sp. *J. Nat. Prod.* **76**, 715–719 (2013).
- 18 Vreys, V. & David, G. Mammalian heparanase: what is the message? *J. Cell Mol. Med.* **11**, 427–452 (2007).

- 19 Meirovitz, A., Goldberg, R., Binder, A., Rubinstein, A. M., Hermano, E. & Elkin, M. Heparanase in inflammation and inflammation-associated cancer. *FEBS J.* **280**, 2307–2319 (2013).
- 20 Sue, M. *et al.* An iminosugar-based heparanase inhibitor heparastatin (SF4) suppresses infiltration of neutrophils and monocytes into inflamed dorsal air pouches. *Int. Immunopharmacol.* **35**, 15–21 (2016).
- 21 Umezawa, H., Aoyagi, T., Komiya, T., Morishima, H. & Hamada, M. Purification and characterization of a sialidase inhibitor, siastatin, produced by *Streptomyces*. *J. Antibiot. (Tokyo)* **27**, 963–969 (1974).
- 22 Rodan, G. A. & Martin, T. J. Therapeutic approaches to bone diseases. *Science* **289**, 1508–1514 (2000).
- 23 Sakamoto, S. *et al.* Decalpenic acid, a novel small molecule from *Penicillium verrucosum* CR37010, induces early osteoblastic markers in pluripotent mesenchymal cells. *J. Antibiot. (Tokyo)* **63**, 703–708 (2010).
- 24 Breyer, R. M., Bagdassarian, C. K., Myers, S. A. & Breyer, M. D. Prostanoid receptors: subtypes and signaling. *Annu. Rev. Pharmacol. Toxicol.* **41**, 661–690 (2001).
- 25 Nakae, K. *et al.* Functional role of prostacyclin receptor in rat dorsal root ganglion neurons. *Neurosci. Lett.* **388**, 132–137 (2005).
- 26 Cathcart, M. C., O'Byrne, K. J., Reynolds, J. V., O'Sullivan, J. & Pidgeon, G. P. COX-derived prostanoid pathways in gastrointestinal cancer development and progression: novel targets for prevention and intervention. *Biochim. Biophys. Acta* **1825**, 49–63 (2012).
- 27 Moreira, L. & Castells, A. Cyclooxygenase as a target for colorectal cancer chemoprevention. *Curr. Drug Targets* **12**, 1888–1894 (2011).
- 28 Ramalho, T. C., Rocha, M., da Cunha, E. F. & Freitas, M. P. The search for new COX-2 inhibitors: a review of 2002–2008 patents. *Expert Opin. Ther.* **9**, 1193–1228 (2009).
- 29 Ng, S. C. & Chan, F. K. NSAID-induced gastrointestinal and cardiovascular injury. *Curr. Opin. Gastroenterol.* **26**, 611–617 (2010).
- 30 Kolbe, L. *et al.* Anti-inflammatory efficacy of Licochalcone A: correlation of clinical potency and *in vitro* effects. *Arch. Dermatol. Res.* **298**, 23–30 (2006).
- 31 Kitanaka, S. *et al.* (+)-Alpha-viniferin, an anti-inflammatory compound from *Caragana chamlagu* root. *Chem. Pharm. Bull. (Tokyo)* **38**, 432–435 (1990).
- 32 Lee, S. H. *et al.* Alpha-viniferin: a prostaglandin H2 synthase inhibitor from root of *Carex humilis*. *Planta Med.* **64**, 204–207 (1998).
- 33 Dilshara, M. G. *et al.* Anti-inflammatory mechanism of alpha-viniferin regulates lipopolysaccharide-induced release of proinflammatory mediators in BV2 microglial cells. *Cell Immunol.* **290**, 21–29 (2014).
- 34 Danz, H. *et al.* Inhibitory activity of tryptanthrin on prostaglandin and leukotriene synthesis. *Planta Med.* **68**, 875–880 (2002).
- 35 Shin, K. M. *et al.* *In vitro* anti-inflammatory activity of 23-hydroxyursolic acid isolated from *Cussonia bancoensis* in murine macrophage RAW 264.7 cells. *Planta Med.* **70**, 803–807 (2004).
- 36 Kim, Y. P. *et al.* Inhibition by tectorigenin and tectoridin of prostaglandin E2 production and cyclooxygenase-2 induction in rat peritoneal macrophages. *Biochim. Biophys. Acta* **1438**, 399–407 (1999).
- 37 Scoditti, E. *et al.* Hydroxytyrosol suppresses MMP-9 and COX-2 activity and expression in activated human monocytes via PKC α and PKC β 1 inhibition. *Atherosclerosis* **232**, 17–24 (2014).
- 38 Cadenas, E. Antioxidant and prooxidant functions of DT-diaphorase in quinone metabolism. *Biochem. Pharmacol.* **49**, 127–140 (1995).
- 39 Nishiyama, T., Izawa, T., Usami, M., Ohnuma, T., Ogura, K. & Hiratsuka, A. Cooperation of NAD(P)H:quinone oxidoreductase 1 and UDP-glucuronosyltransferases reduces menadione cytotoxicity in HEK293 cells. *Biochem. Biophys. Res. Commun.* **394**, 459–463 (2010).
- 40 Murakami, M. *et al.* Signaling of vascular endothelial growth factor receptor-1 tyrosine kinase promotes rheumatoid arthritis through activation of monocytes/macrophages. *Blood* **108**, 1849–1856 (2006).
- 41 De Bandt, M. *et al.* Blockade of vascular endothelial growth factor receptor I (VEGF-RI), but not VEGF-RII, suppresses joint destruction in the K/BxN model of rheumatoid arthritis. *J. Immunol.* **171**, 4853–4859 (2003).
- 42 Carmeliet, P. & Jain, R. K. Angiogenesis in cancer and other diseases. *Nature* **407**, 249–257 (2000).
- 43 Saaristo, A. *et al.* Vascular endothelial growth factor-C and its receptor VEGFR-3 in the nasal mucosa and in nasopharyngeal tumors. *Am. J. Pathol.* **157**, 7–14 (2000).
- 44 Karkkainen, M. J. & Petrova, T. V. Vascular endothelial growth factor receptors in the regulation of angiogenesis and lymphangiogenesis. *Oncogene* **19**, 5598–5605 (2000).
- 45 Nakajima, M., Irimura, T., Di Ferrante, D., Di Ferrante, N. & Nicolson, G. L. Heparan sulfate degradation: relation to tumor invasive and metastatic properties of mouse B16 melanoma sublines. *Science* **220**, 611–613 (1983).
- 46 Naparstek, Y., Cohen, I. R., Fuks, Z. & Vlodavsky, I. Activated T lymphocytes produce a matrix-degrading heparan sulphate endoglycosidase. *Nature* **310**, 241–244 (1984).
- 47 Matzner, Y., Bar-Ner, M., Yahalom, J., Ishai-Michaeli, R., Fuks, Z. & Vlodavsky, I. Degradation of heparan sulfate in the subendothelial extracellular matrix by a readily released heparanase from human neutrophils. Possible role in invasion through basement membranes. *J. Clin. Invest.* **76**, 1306–1313 (1985).
- 48 Fridman, R., Lider, O., Naparstek, Y., Fuks, Z., Vlodavsky, I. & Cohen, I. R. Soluble antigen induces T lymphocytes to secrete an endoglycosidase that degrades the heparan sulfate moiety of subendothelial extracellular matrix. *J. Cell Physiol.* **130**, 85–92 (1987).
- 49 Vlodavsky, I. *et al.* Expression of heparanase by platelets and circulating cells of the immune system: possible involvement in diapedesis and extravasation. *Invasion Metastasis* **12**, 112–127 (1992).
- 50 Lider, O. *et al.* Suppression of experimental autoimmune diseases and prolongation of allograft survival by treatment of animals with low doses of heparins. *J. Clin. Invest.* **83**, 752–756 (1989).
- 51 Lider, O. *et al.* Inhibition of T lymphocyte heparanase by heparin prevents T cell migration and T cell-mediated immunity. *Eur. J. Immunol.* **20**, 493–499 (1990).
- 52 Yahalom, J., Fibach, E., Bar-Tana, R., Fuks, Z. & Vlodavsky, I. Differentiating human leukemia cells express heparanase that degrades heparan sulfate in subendothelial extracellular matrix. *Leuk. Res.* **12**, 711–717 (1988).
- 53 Li, J. P. & Vlodavsky, I. Heparin, heparan sulfate and heparanase in inflammatory reactions. *Thromb. Haemost.* **102**, 823–828 (2009).
- 54 Morris, A. *et al.* The role of heparanase in pulmonary cell recruitment in response to an allergic but not non-allergic stimulus. *PLoS ONE* **10**, e0127032 (2015).
- 55 Benhamron, S. *et al.* Dissociation between mature phenotype and impaired transmigration in dendritic cells from heparanase-deficient mice. *PLoS ONE* **7**, e35602 (2012).
- 56 Poon, I. K. *et al.* Mice deficient in heparanase exhibit impaired dendritic cell migration and reduced airway inflammation. *Eur. J. Immunol.* **44**, 1016–1030 (2014).
- 57 Sasaki, N. *et al.* Cell surface localization of heparanase on macrophages regulates degradation of extracellular matrix heparan sulfate. *J. Immunol.* **172**, 3830–3835 (2004).
- 58 Stoler-Barak, L. *et al.* Heparanase of murine effector lymphocytes and neutrophils is not required for their diapedesis into sites of inflammation. *FASEB J.* **29**, 2010–2021 (2015).
- 59 Willenborg, D. O. & Parish, C. R. Inhibition of allergic encephalomyelitis in rats by treatment with sulfated polysaccharides. *J. Immunol.* **140**, 3401–3405 (1988).
- 60 Willenborg, D. O. & Parish, C. R. Inhibition of passive allergic encephalomyelitis by sulfated polysaccharides. *Ann. N. Y. Acad. Sci.* **540**, 543–545 (1988).
- 61 Parish, C. R., Hindmarsh, E. J., Bartlett, M. R., Staykova, M. A., Cowden, W. B. & Willenborg, D. O. Treatment of central nervous system inflammation with inhibitors of basement membrane degradation. *Immunol. Cell Biol.* **76**, 104–113 (1998).
- 62 Edovitsky, E., Lerner, I., Zcharia, E., Peretz, T., Vlodavsky, I. & Elkin, M. Role of endothelial heparanase in delayed-type hypersensitivity. *Blood* **107**, 3609–3616 (2006).
- 63 Floor, M. *et al.* Enoxaparin improves the course of dextran sodium sulfate-induced colitis in syndecan-1-deficient mice. *Am. J. Pathol.* **176**, 146–157 (2010).
- 64 Nishimura, Y. *et al.* Flexible synthesis and biological activity of uronic acid-type gem-diamine 1-N-iminosugars: a new family of glycosidase inhibitors. *J. Org. Chem.* **65**, 2–11 (2000).
- 65 Nishimura, Y. *et al.* Effect on spontaneous metastasis of mouse Lewis lung carcinoma by a trifluoroacetamide analogue of siastatin B. *J. Antibiot. (Tokyo)* **47**, 840–842 (1994).
- 66 Nishimura, Y. *et al.* Totally synthetic analogues of siastatin B. III. Trifluoroacetamide analogues having inhibitory activity for tumor metastasis. *J. Antibiot. (Tokyo)* **47**, 101–107 (1994).
- 67 Satoh, T. *et al.* Synthesis and antimetastatic activity of 6-trichloroacetamido and 6-guanidino analogues of siastatin B. *J. Antibiot. (Tokyo)* **49**, 321–325 (1996).
- 68 Toyoshima, M. & Nakajima, M. Human heparanase. Purification, characterization, cloning, and expression. *J. Biol. Chem.* **274**, 24153–24160 (1999).
- 69 Loi, F., Cordova, L. A., Pajarinen, J., Lin, T. H., Yao, Z. & Goodman, S. B. Inflammation, fracture and bone repair. *Bone* **86**, 119–130 (2016).
- 70 Wu, X., Ding, S., Ding, Q., Gray, N. S. & Schultz, P. G. A small molecule with osteogenesis-inducing activity in multipotent mesenchymal progenitor cells. *J. Am. Chem. Soc.* **124**, 14520–14521 (2002).
- 71 Wu, X., Walker, J., Zhang, J., Ding, S. & Schultz, P. G. Purmorphamine induces osteogenesis by activation of the hedgehog signaling pathway. *Chem. Biol.* **11**, 1229–1238 (2004).
- 72 Beloti, M. M., Bellesini, L. S. & Rosa, A. L. Purmorphamine enhances osteogenic activity of human osteoblasts derived from bone marrow mesenchymal cells. *Cell Biol. Int.* **29**, 537–541 (2005).
- 73 Sinha, S. & Chen, J. K. Purmorphamine activates the Hedgehog pathway by targeting Smoothened. *Nat. Chem. Biol.* **2**, 29–30 (2006).
- 74 Zhou, S. *et al.* Estrogens activate bone morphogenetic protein-2 gene transcription in mouse mesenchymal stem cells. *Mol. Endocrinol.* **17**, 56–66 (2003).
- 75 Li, X. *et al.* Identification of upregulators of BMP2 expression via high-throughput screening of a synthetic and natural compound library. *J. Biomol. Screen.* **14**, 1251–1256 (2009).
- 76 Jin, P., Wu, H., Xu, G., Zheng, L. & Zhao, J. Epigallocatechin-3-gallate (EGCG) as a pro-osteogenic agent to enhance osteogenic differentiation of mesenchymal stem cells from human bone marrow: an *in vitro* study. *Cell Tissue Res.* **356**, 381–390 (2014).
- 77 Yamaguchi, M., Arbisser, J. L. & Weitzmann, M. N. Honokiol stimulates osteoblastogenesis by suppressing NF- κ B activation. *Int. J. Mol. Med.* **28**, 1049–1053 (2011).
- 78 Yamaguchi, A. Regulation of differentiation pathway of skeletal mesenchymal cells in cell lines by transforming growth factor-beta superfamily. *Semin. Cell Biol.* **6**, 165–173 (1995).
- 79 Hata, K. *et al.* Differential roles of Smad1 and p38 kinase in regulation of peroxisome proliferator-activating receptor gamma during bone morphogenetic protein 2-induced adipogenesis. *Mol. Biol. Cell* **14**, 545–555 (2003).
- 80 Makhijani, N. S., Bischoff, D. S. & Yamaguchi, D. T. Regulation of proliferation and migration in retinoic acid treated C3H10T1/2 cells by TGF-beta isoforms. *J. Cell Physiol.* **202**, 304–313 (2005).
- 81 Wiper-Bergeron, N., St-Louis, C. & Lee, J. M. CCAAT/Enhancer binding protein beta abrogates retinoic acid-induced osteoblast differentiation via repression of Runx2 transcription. *Mol. Endocrinol.* **21**, 2124–2135 (2007).

- 82 Wang, A., Ding, X., Sheng, S. & Yao, Z. Retinoic acid inhibits osteogenic differentiation of rat bone marrow stromal cells. *Biochem. Biophys. Res. Commun.* **375**, 435–439 (2008).
- 83 Zhang, W. *et al.* Retinoic acids potentiate BMP9-induced osteogenic differentiation of mesenchymal progenitor cells. *PLoS ONE* **5**, e11917 (2010).
- 84 Sakamoto, S., Kojima, F., Momose, I., Kawada, M., Adachi, H. & Nishimura, Y. Decalpenic acid induces early osteoblastic markers in pluripotent mesenchymal cells via activation of retinoic acid receptor gamma. *Biochem. Biophys. Res. Commun.* **422**, 751–757 (2012).
- 85 Bastien, J. & Rochette-Egly, C. Nuclear retinoid receptors and the transcription of retinoid-target genes. *Gene* **328**, 1–16 (2004).
- 86 Germain, P. *et al.* International Union of Pharmacology. LXIII. Retinoid X receptors. *Pharmacol. Rev.* **58**, 760–772 (2006).