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Identification of a naturally occurring retinoid X receptor agonist from Brazilian green propolis

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ABSTRACT

Background: Brazilian green propolis (BGP), a resinous substance produced from *Baccharis dracunculifolia* by Africanized honey bees (*Apis mellifera*), is used as a folk medicine. Our present study explores the retinoid X receptor (RXR) agonistic activity of BGP and the identification of an RXR agonist in its extract.
Methods: RXRα agonistic activity was evaluated using a luciferase reporter gene assay. Isolation of the RXRα agonist from the ethanolic extract of BGP was performed using successive silica gel and a reversed phase column chromatography. The interaction between the isolated RXRα agonist and RXRα protein was predicted by a receptor–ligand docking simulation. The nuclear receptor (NR) cofactor assay was used to estimate whether the isolated RXRα agonist bound to various NRs, including RXRs and peroxisome proliferator-activated receptors (PPARs). We further examined its effect on adipogenesis in 3T3-L1 fibroblasts.
Results: We identified drupanin as an RXRα agonist with an EC_{50} value of 4.8 ± 1.0 μM. Drupanin activated three RXR subtypes by a similar amount and activated PPARγ moderately. Additionally, drupanin induced adipogenesis and elevated aP2 mRNA levels in 3T3-L1 fibroblasts.
Conclusions: Drupanin, a component of BGP, is a novel RXR agonist with slight PPARγ agonistic activity.
General significance: This study revealed for the first time that BGP activates RXR and drupanin is an RXR agonist in its extract.

1. Introduction

The retinoid X receptor (RXR), which is classified as a subfamily 2 nuclear receptor (NR), forms either homodimers or heterodimers with subfamily 1 NRs, such as peroxisome proliferator-activated receptors (PPARs), retinoic acid receptors (RARs), liver X receptors (LXRs), farnesoid X receptor, vitamin D receptor (VDR), and thyroid hormone receptors [1]. These heterodimers have been further subclassified as permissive or non-permissive heterodimers [2–5]. Permissive heterodimers formed by RXRs and partner NRs, such as PPARs, LXRs, and farnesoid X receptor, are synergistically activated by RXR-specific and partner NR-specific ligands. Conversely, non-permissive heterodimers formed by RARs and VDR can only be activated by ligands that are specific for the partner NR. RXRs are regulators of various pathophysiological processes with potential clinical implications. For instance, synthetic RXR ligands exert beneficial glucose-lowering and insulin-sensitizing effects as well as antiobesity actions in animal models of insulin-resistance and diabetes. They also improve cholesterol homeostasis and inhibit the development of atherosclerosis in a mouse model of mixed dyslipidemia [6,7]. Although there have been a considerable number of studies on synthetic RXR ligands, and many agonists have been reported, naturally occurring ones are rare, except for a few examples [8,9]. Our ongoing studies of RXR ligands in natural materials have resulted in the identification of honokiol and magnolol as RXR agonistic components from the bark of *Magnolia obovata* [10,11].

Propolis is made from resinous plant substances gathered by honey bees (*Apis* spp.) and is used to cement the opening of the hive. There are various theories as to the role of propolis; however, it is generally understood that it plays important roles in protecting bees from enemies and microbes, fixing the hive, and maintaining the temperature of the hive. The chemical constituents of propolis differ substantially from region to region and season to season because of its dependency on the raw plant material. In particular, Brazilian green propolis (BGP), which is mainly gathered from Minas Gerais in Brazil, exhibits the characteristic constituents of propolis from other districts. Kumazawa et al. identified the plant origin of BGP as an asteraceous plant, *Baccharis dracunculifolia*, by observing honey bee behavior and performing phytochemical analysis [12]. As a folk medicine, BGP is used for its anti-inflammatory and antimicrobial properties [13]. Previous studies on the biological activity of BGP extract have revealed its efficacy for the treatment of inflammation, malignancy, and obesity [14–16]. The

Abbreviations: BGP, Brazilian green propolis; FBS, fetal bovine serum; HEK, human embryonic kidney; LBD, ligand binding domain; LXR, liver X receptor; NR, nuclear receptor; PPAR, peroxisome proliferator-activated receptor; RAR, retinoic acid receptor; RXR, retinoid X receptor; TLC, thin layer chromatography; VDR, vitamin D receptor

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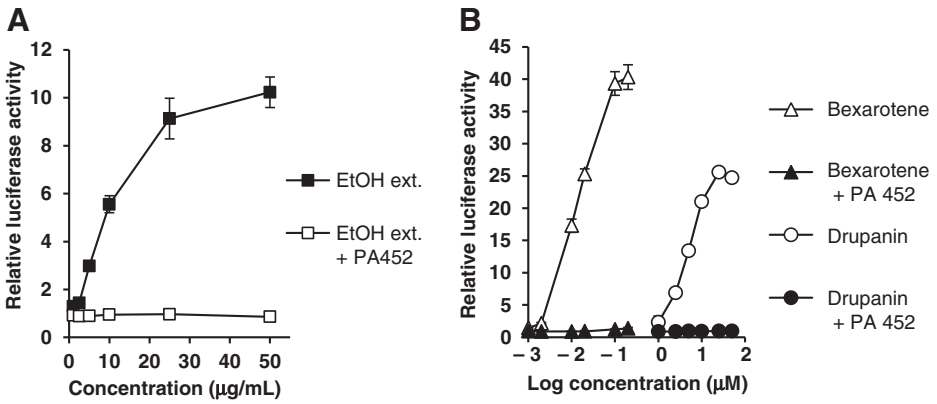


Fig. 1. RXRα agonistic activity of an ethanolic extract of BGP (A) and drupanin (B) in the luciferase reporter assay. HEK293 cells were co-transfected with the RXRα expression vectors and luciferase reporter plasmids together with pCMX β-gal, as described in Section 2.4. At 6 h after transfection, the cells were treated with increasing concentrations of EtOH ext. (open squares), drupanin (**1**) (open circles), and bexarotene (open triangles) with/without 10 μM PA452 (RXR antagonist; closed circles) for 48 h. Data are represented as the mean ± SD of three determinants.

chemical constituents of BGP are rich in cinnamic acid derivatives bearing 3,3-dimethylallyl (prenyl) groups. Artepillin C, which is a major biologically active component of BGP, possesses antibacterial, antitumor, and anti-inflammatory activity [17–19]. Recently, it was reported that artepillin C showed agonistic activity for PPARγ, which is involved in the regulation of obesity, inflammation, and cancer [20,21]. However, we found that the PPARγ agonistic activity of artepillin C was considerably weaker than that of synthetic agonists (unpublished data). Therefore, it is not likely to contribute to the beneficial effects of BGP. Thus, we inferred the involvement of RXR in the molecular mechanism underlying the effects of BGP. Herein, we describe the identification of an RXR agonist from BGP and its adipogenic effect on adipocytes to provide a better understanding of the mechanism of its biological activity.

2. Materials and methods

2.1. Chemicals, reagents, and general experimental procedure

The NR agonists bexarotene, WY14643, and T0901317 were purchased from Cayman Chemical Co. (Ann Arbor, MI). Rosiglitazone and GW501516 were purchased from Enzo Life Sciences (Farmingdale, NY). *All-trans* retinoic acid was purchased from Sigma-Aldrich (St. Louis, MO). The RXR antagonist PA452 was kindly provided by Dr. Hiroyuki Kagechika (Tokyo Medical and Dental University, Tokyo, Japan). Artepillin C, which was used in a preliminary test, was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Silica gel AP-300 purchased from Toyota Kako (Aichi, Japan), Sephadex LH-20 purchased from GE Healthcare (Uppsala, Sweden), and Cosmosil $75C_{18}$-OPN purchased from Nacalai Tesque (Kyoto, Japan) were used for column chromatography. Silica gel 60 F_{254} and silica gel RP-18 F_{254S}, which were purchased from Merck (Darmstadt, Germany), were used for thin layer chromatography (TLC). Nuclear magnetic resonance spectra were recorded on a JNM-AL-400 spectrometer (JEOL, Tokyo, Japan) with tetramethylsilane as the internal standard.

2.2. Isolation and structure elucidation

The ethanolic extract of BGP was purchased from Nihon Yoho (Gifu, Japan; 166 g). The extract was subjected to SiO_2 column purification (*n*-C_6H_{14}/Me_2CO (8:1 → 1:1, each 5 L) in stepwise gradient mode and fractions of 1 L were collected. According to their TLC profiles, the resulting fractions were combined into 14 fractions: Fr. 1–2 (*n*-C_6H_{14}/Me_2CO, 8:1), Fr. 3–4 (6:1), Fr. 5–7 (4:1), Fr. 8–10 (2:1), Fr. 11–13 (1:1), Fr. 14 (0:1). Fr. 9 was dissolved in EtOH and applied to a mixture of beturetol and kaempferide, and then the filtrate was purified by ODS column chromatography using Cosmosil $75C_{18}$-OPN in an isocratic solvent system (MeCN/H_2O, 2:3) to yield drupanin (**1**; 3.4 g). Artepillin C (**2**; 1.62 g), (2*E*)-3-(2,2-dimethyl-*2H*-1-benzopyran-6-yl)-2-propenoic acid (**3**; 17.5 mg), (2*E*)-3-[3,4-dihydro-3-hydroxy-2,2-dimethyl-8-(3-methyl-2-buten-1-yl)-*2H*-1-benzopyran-6-yl]-2-propenoic acid (**4**; 15.2 mg), and (2*E*)-3-[7-(3-methyl-2-buten-1-yl)-2-(1-methylethenyl)-5-benzofuranyl]-2-propenoic acid (**5**; 47.9 mg) were isolated from part of Fr. 8 with the aid of Sephadex LH-20 (MeOH), an ODS column using Cosmosil $75C_{18}$-OPN and an isocratic solvent system (MeCN/H_2O, 2:3), and preparative TLC ($CHCl_3$/EtOH, 20:1). The structures of the isolated compounds were determined by comparison with ^1H and ^{13}C NMR spectral data in the literature [22] (Table S1).

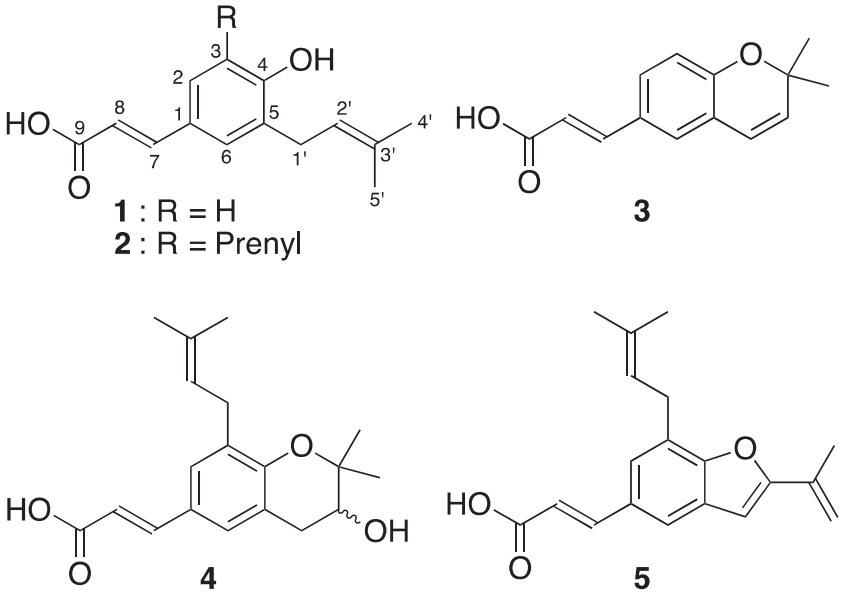
2.3. Cell culture

Human embryonic kidney (HEK) 293 cells were maintained in minimum essential medium supplemented with 10% fetal bovine serum (FBS), 1% nonessential amino acids (Sigma-Aldrich), 50 U/mL penicillin, and 50 μg/mL streptomycin at 37 °C in a humidified atmosphere of 5% CO_2 in air. 3T3-L1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum, 1%, 50 U/mL penicillin, and 50 μg/mL streptomycin at 37 °C in a 5% CO_2 humidified atmosphere.

2.4. Luciferase reporter gene assay

HEK293 cells were co-transfected with the NR expression vectors and luciferase reporter plasmids together with the pCMX β-gal expression vector and carrier DNA pUC18, as described previously [10,23].

Briefly, HEK293 cells were co-transfected by calcium phosphate coprecipitation with pCMX-hRAR-α (30 ng) and tk-βRE-Luc (120 μg) for the RAR luciferase reporter assay; pBApo-CMX-hLXR-α (30 ng) and pGL4.1-DR4-Luc (120 ng) for the LXR luciferase reporter assay; and pBIND-VDR (30 ng) and pG5-Luc (120 ng), in addition to pCMX-β-gal expression vector (30 ng) and carrier DNA pUC18 to yield a total of 600 ng of DNA per well. At 6 h after transfection, the cells were treated for another 48 h with the test samples at the indicated concentrations in medium containing 10% FBS. The luciferase and β-galactosidase activities of cell lysates were analyzed using a luminescence reader and a spectrophotometer, respectively. Luciferase activity was normalized by β-gal and expressed as fold inductions relative to that in vehicle-treated cells. The data represent the means ± SD of three determinants from a representative of three independent experiments that showed similar results.



Scheme 1. Structures of the isolated constituents from BGP (**1**–**5**).

2.5. Receptor–ligand docking simulation

The CDOCKER algorithm with a CHARMm force field in Discovery Studio 3.1 (Accelrys, San Diego, CA) was used to simulate receptor–ligand interactions. The starting X-ray crystal structures in RXRα of the intact PPARγ-RXRα NR complex bound to each ligand (PDB ID: 1K74, 3DZY, and 1FM6) were obtained from the RCSB protein data bank (http://www.rcsb.org/pdb/). Prior to docking, the RXRα and ligands' structures were prepared using CHARMm force field. The ligand-binding domain (LBD) was defined as the binding site of the internal ligand by using the receptor cavity finding protocol in Discovery Studio, and docking the ligands to LBD. During the docking simulation, all the parameters were the default settings, although the orientations to refine were changed from 10 to 30 and the number of random conformations was increased from 10 to 30.

2.6. Selectivity for RXR and PPAR subtypes

The binding activity for each RXR and PPAR subtype was examined by NR cofactor assays using EnBio RCAS kits for RXRs and PPARs (Fujikura Kasei, Tokyo, Japan) according to the manufacturer's instructions as previously described [24]. The binding activity in this kit is based on the ligand-dependent interaction between RXR isotypes and the steroid receptor coactivator-1 (SRC-1), or PPAR isotypes and the cAMP responsive element-binding protein–binding protein (CBP). Detection relies on anti-RXR and anti-PPAR antibodies conjugated with horseradish peroxidase. Briefly, biotinylated coactivator peptide solution was added to the wells of a 96-well plate coated with solid phase avidin and incubated overnight at 4 °C. After washing thoroughly, the mixture of recombinant receptor and test samples (drupanin and each nuclear receptor agonist) was added and incubated for 1 h at room temperature. After washing three times with wash buffer, the horseradish peroxidase-conjugated antibodies against respective NRs were added and incubated for 1.5 h at room temperature. The plate was then washed and the enzyme reaction was started by the addition of tetramethylbenzidine solution (100 μL) and was allowed to run for 20 min. The absorbance at 450 nm (A_{450}) was measured with a microplate reader. CD3254 (RXRs), GW7647 (PPARα), GW1929 (PPARγ), and GW501516 (PPARδ) were used as the full agonists. The activity relative to the maximum activity of the full agonist (B/B_{max}) was expressed as the percentage of the difference between the A_{450} values of the test sample and the vehicle divided by the difference between the A_{450} values of the maximum value of the full agonist and of the vehicle.

2.7. Oil red O staining

Adipogenic differentiation of 3T3-L1 cells was carried out as follows. DMEM containing 50 ng/mL insulin, 1 μM dexamethasone, 500 μM 1-methyl-3-isobutylxanthine, 10% FBS, and the test samples was added 2 days after the cells reached confluency (day 0). The medium was replaced with DMEM containing 50 ng/mL insulin and the test samples at days 2 and 4. Six days after the start of differentiation, 3T3-L1 cells were fixed for 1 h with 10% formalin in phosphate-buffered saline, washed with 60% isopropanol, and stained with Oil red O for 15 min. The cells were rinsed three times with water and were photomicrographed using a phase-contrast microscope.

2.8. Quantitative RT-PCR analysis

Total RNA was isolated from the cultured cells using RNAiso Plus (Takara Bio, Inc., Shiga, Japan). After treatment of the RNA samples with DNase, first strand cDNA was reverse-transcribed from 0.5 μg total RNA using a ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan). Quantitative real-time PCR analysis was performed using the TP-800 Thermal Cycler Dice real-time system (Takara Bio, Inc.) with SYBR Green. The expression levels of β-actin mRNA were used as a reference for normalization of the mRNA expression levels of the target genes using the quantitation comparative Ct method. The primer sequences used in the present study were as follows: β-actin forward 5′-CAT CCG TAA AGA CCT CTA TGC CAA C-3′, β-actin reverse 5′-ATG GAG CCA CCG ATC CAC A-3′, aP2 forward 5′-AAA TCA CCG CAG ACG ACA GG-3′, and aP2 reverse 5′-TCC ATC CCA CTT CTG CAC CT-3′.

2.9. Statistical analysis

Data are presented as the mean ± standard deviation (SD) and were evaluated for statistical significance by one-way analysis of variance, followed by Bonferroni's method. EC_{50} values were calculated by R software (drc add-on package).

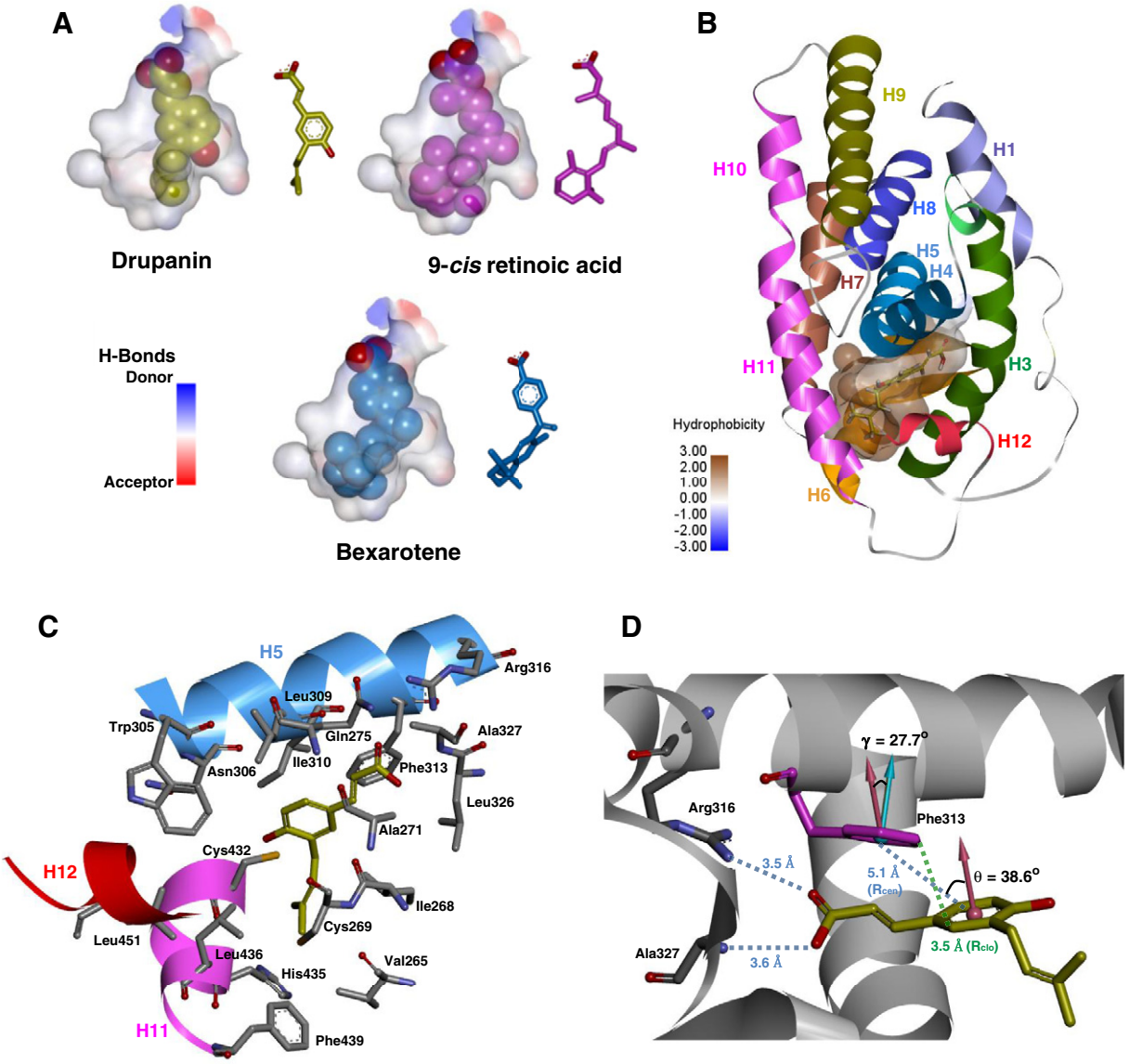


Fig. 2. Docked structural models of the binding of drupanin to RXRα (PDB ID: 1K74) and comparison with binding of other ligands. The docked models were acquired by the method described in Section 2.5. (A) Docking results of drupanin and bexarotene with RXRα and crystal holo-structure of 9-*cis* retinoic acid (PDB ID: 1K74). Surface representation of hydrogen bond donating (blue) or accepting (red) properties. (B) Overall docked structure of RXRα LBD with drupanin. Surface representation of hydrophobic (brown) and hydrophilic (blue) areas. (C) Docking results of drupanin and amino acid residues that were located within 4 Å of the ligand. (D) Plausible intermolecular interactions between drupanin (yellow) and RXRα, and orientational information. We used the intermolecular orientational information for the aromatic interaction with Phe313 (magenta) reported by McGaughey et al. [40]. Centroid-centroid separation (R_{cen}), closest contact distance (R_{clo}), a center-normal angle (θ), and normal-normal angle (γ).

3. Results

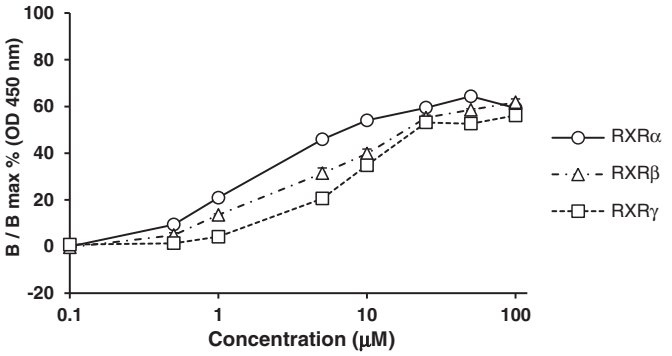
3.1. Ethanolic extract of BGP shows RXRα agonistic activity

In the RXRα luciferase reporter gene assay, the ethanolic extract of BGP increased luciferase activity (Fig. 1A). When the extract was treated with PA452, an RXR antagonist, the activity was completely abolished. Furthermore, artepillin C, which is a well-known active component of BGP, failed to show activity for RXRα (Supplemental data, Fig. S1).

3.2. Identification of drupanin as a component that activates RXRα

On the basis of the results for the crude extract, we sought to isolate the RXR agonist from BGP using a bioassay-guided isolation technique. The ethanolic extract of the BGP was subjected to SiO_2 column purification to afford 14 fractions (Frs. 1–14). Among these fractions, only Frs. 9 and 10 showed biological activity. Drupanin (**1**) was isolated from Fr. 9 by the purification procedure described in Section 2.2. Drupanin, a monoprenylated cinnamic acid derivative, had significant RXRα agonistic activity in a dose-dependent manner with an EC_{50} value of 4.8 ± 1.0 μM and an efficacy rate of approximately 62.5 ± 1.2% that of bexarotene (EC_{50} value, 14.0 ± 1.0 nM) in the luciferase reporter gene assay (Fig. 1B). Furthermore, PA452 completely inhibited the increase of luciferase activity by drupanin (Fig. 1B).

Additionally, artepillin C (**2**), (2*E*)-3-(2,2-dimethyl-2*H*-1-benzopyran-6-yl)-2-propenoic acid (**3**), (2*E*)-3-[3,4-dihydro-3-hydroxy-2,2-dimethyl-8-(3-methyl-2-buten-1-yl)-2*H*-1-benzopyran-6-yl]-2-propenoic acid (**4**), and (2*E*)-3-[7-(3-methyl-2-buten-1-yl)-2-(1-methylethenyl)-5-benzofuranyl]-2-propenoic acid (**5**) were isolated from Fr. 8 (Scheme 1), all of which showed no RXRα-activating effect (data not shown).



subtypes	RXRα	RXRγ	RXRδ
EC_{50} (μM)[a]	2.1 ± 0.1	4.6 ± 0.3	7.0 ± 0.3
%Efficacy[b]	64.4 ± 1.1	61.8 ± 1.5	56.1 ± 1.8

[a] EC_{50} values were the concentration when B/Bmax % was 50%.

[b] %Efficacy values were maximum B/Bmax %.

Fig. 3. Effect of drupanin on the recruitment of the cofactor for each RXR subtype in the NR cofactor assay. The recruitment of steroid receptor coactivator (SRC) at various drupanin concentrations was evaluated, as described in Section 2.6. B/B_{max} was expressed as the percentage of the difference between the A_{450} values of the test sample and the vehicle, divided by the difference between the A_{450} values of the maximum value of the full agonist and the vehicle. Values are given as the percentage of the relative A_{450} to the maximum A_{450} of the positive control. Data are represented as the mean ± SD of three determinants.

3.3. Binding mode of drupanin with RXRα

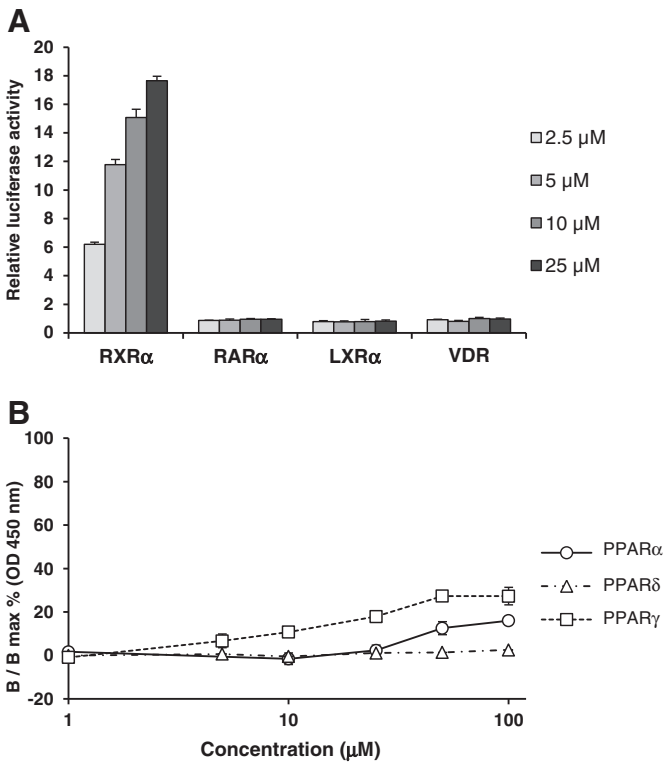
We performed a receptor–ligand docking simulation to estimate the molecular interaction of drupanin with RXRα by comparison with the interactions of other RXR ligands. After calculating the binding of the RXRα holo-structure (PDB ID: 1K74) with drupanin, 9-*cis* retinoic acid, and bexarotene, docking results were obtained with all the tested ligands (Fig. 2A). The docking root-mean-square deviation (RMSD) between docked and co-crystallized 9-*cis* retinoic acid was 0.75, thus the docking simulation was reliable. Additionally, we confirmed that similar results could be acquired using other RXRα crystal holo-structures (PDB ID: 3DZY and 1FM6), which also suggested that the acquired docking results were reliable. The CDOCKER interaction energies of drupanin, 9-*cis* retinoic acid, and bexarotene were −35.6, −48.4, and −51.7, respectively. Fig. 2C and D shows that the carboxyl group was located close to the guanidino group of Arg316 and the amino group of Ala327, and the aromatic ring interacted with Phe313 by π–π stacking. Furthermore, a prenyl group, which has hydrophobic properties, fits into the highly hydrophobic space (Fig. 2B).

3.4. Selectivity of drupanin for RXR subtypes

To investigate whether drupanin binds selectively to an RXR subtype, an NR cofactor assay for each RXR subtype was performed. This assay detects the recruitment of a solid-phased cofactor to RXRs, following the binding of ligands to RXRs, using antibodies against RXRs. Drupanin exhibited a dose-dependent increase in cofactor recruitment to each subtype (Fig. 3).

3.5. Activation of subfamily 1 NRs by drupanin

NR agonists often activate multiple NRs; for instance, magnolol activates RXRα and PPARγ, and 9-*cis* retinoic acid activates RXRα and RARα [25,26]. Thus, we examined the selectivity of drupanin for subfamily 1 NRs using luciferase reporter gene assays. As shown in Fig. 4A, drupanin slightly increased luciferase activity for each PPAR subtype (α, γ, and δ); however, it failed to increase activity in RARα, LXRα, and VDR luciferase reporter gene assays. We also performed the NR cofactor assay for each



subtypes	PPARα	PPARγ	PPARδ
EC_{50} (μM)[a]	39.0 ± 3.7	14.6 ± 1.4	N.D.[c]
%Efficacy[b]	17.0 ± 2.9	29.5 ± 2.8	2.0 ± 1.4

[a] EC_{50} values were the concentration when B/Bmax % was 50%.

[b] %Efficacy values were maximum B/Bmax %.

[c] N.D. is not detected.

Fig. 4. Selectivity of drupanin for subfamily 1 NRs. (A) The cells were co-transfected with the respective NR expression vector and luciferase reporter plasmids as described in Section 2.4. Activity was normalized using β-gal and expressed as fold induction relative to that of vehicle-treated cells. (B) Effect of drupanin on the recruitment of the cofactor for each PPAR subtype in the NR cofactor assay. The recruitment of CBP for each RXR subtype at various concentrations of drupanin was evaluated as described in Section 2.6. B/B_{max} was expressed as the percentage of the difference between the A_{450} values of the test sample and the vehicle divided by the difference between the A_{450} values of the maximum value of the full agonist and the A_{450} of the vehicle. Values are given as the percentage of the relative A_{450} to maximum A_{450} of positive control. Data are represented as the mean ± SD of three determinants.

PPAR subtype (α, γ, and δ). The NR cofactor assay for PPARs can detect the binding of ligands to PPARs indirectly by measuring the ligand-dependent interaction between PPARs and the coactivator CBP. Drupanin was found to stimulate the interaction between PPARγ and CBP in a dose-dependent manner at relatively low concentrations (EC_{50} value: 14.6 ± 1.4 μM), where a physiological effect may be expected (Fig. 4B). In the case of PPARα, drupanin activated the interaction with CBP only at high concentrations and with low efficacy, where the physiological effects of NR ligands are usually ineffective. However, drupanin failed to activate PPARδ.

3.6. Adipogenic effect of drupanin on 3T3-L1 cells

PPARγ agonists promote the differentiation and adipogenesis of preadipocytes to produce and secrete more adipocytokines such as adiponectin. As adiponectin enhances insulin sensitivity and inhibits the elevation of blood sugar levels, thiazolidine derivatives, which are well-known PPARγ agonists, are used widely for the treatment of insulin-resistant type 2 diabetes. We tested whether drupanin enhanced the adipogenesis of mouse 3T3-L1 fibroblasts. As shown by Oil

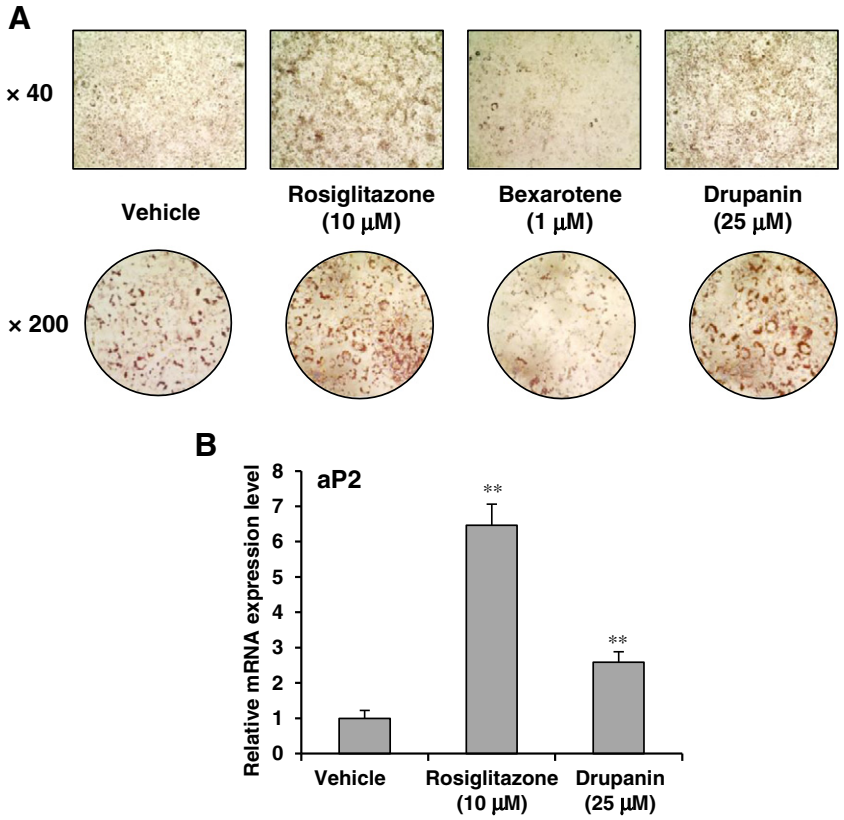


Fig. 5. Effect of drupanin on the differentiation and adipogenesis of 3T3-L1 pre-adipocytes. (A) Oil red O staining of differentiated pre-adipocytes with each treatment on day 6 and photographed under a phase-contrast microscope. Original magnification of the above squares and below circles is × 40 and × 200, respectively. (B) The gene transcription level of aP2 was measured by RT-qPCR on day 5, and expressed as a ratio to β-actin. Results represent the mean ± SD of two independent experiments, each performed using quadruplicate wells. $^{**}p < 0.01$, compared with the vehicle control.

red O staining on day 6, drupanin significantly promoted the accumulation of lipid droplets (Fig. 5A). On day 5 after starting differentiation, the mRNA levels of aP2, which is a target gene of PPARγ and a marker of adipogenesis, were significantly enhanced (Fig. 5B).

4. Discussion

Our ongoing studies to identify RXR agonists derived from natural products led to the discovery that BGP shows an RXRα agonistic effect (Fig. 1A). As RXR forms a heterodimer with partner NRs belonging to various subfamily 1 NRs and regulates the transcriptional activity of RXR heterodimers, we hypothesized that the multiple functions of BGP may, at least in part, be explained by the involvement of RXR. We preliminarily confirmed that artepillin C, which has been identified as a PPARγ agonist, is unable to activate RXRα [20,21]. A number of cinnamic acid derivatives bearing moieties derived from the prenyl group have been isolated [27–29]. In the present study, five known cinnamic acid derivatives were isolated. RXRα agonistic activity was exhibited only by drupanin (Fig. 1B).

We conducted receptor–ligand docking simulations to predict binding orientations, and acquired the docked structures of RXRα/drupanin, RXRα/bexarotene, and RXRα/9-*cis* retinoic acid (Fig. 2A). Drupanin occupied a similar ligand binding pocket and fitted well in a similar binding mode to existing RXR agonists [30,31]. The interaction of the carboxylic group to Arg316 and Ala327 and the π–π stacking between the aromatic ring in drupanin and Phe313 might be contributed to high affinity for RXRα (Fig. 2D). The competition experiments between drupanin and 9-*cis* retinoic acid in the luciferase reporter assay also suggested that drupanin occupied the same LBD as 9-*cis* retinoic acid (Fig. S2). Furthermore, structural studies of NRs suggested that the C-terminal helix H12 of the receptor plays a major role in generating the surface for coactivator binding or recognition in its ligand-induced functional conformation. The binding of agonists to NRs triggers a conformational change in the ligand binding domain (LBD) with the repositioning of H12, creating a binding surface that allows a coactivator to bind. In contrast, antagonists prevent H12 from adopting its active conformation, facilitating the interaction with corepressors [32]. In the RXR LBD, crystallographic studies showed that Leu436 in H11 is conformationally changed by the steric interaction with the RXR antagonist and this prevents H12 from adopting the active conformation because of a steric clash with Leu455 in H12 [33,34]. Thus, it was suggested that the steric interactions between the ligand and the ligand binding cavity residues, particularly residues close to H12, gave rise to the agonistic behavior. Although the prenyl group of drupanin points toward the hydrophobic cavity near H12, which is formed by residues Val265, Cys269, His435, Leu436, and Phe439, it fits in the ligand binding cavity well without steric effects (Fig. 2C). Therefore, H12 was presumably able to adopt an active conformation in the RXRα/drupanin complex, and drupanin may function as an RXR agonist.

RXR is classified into 3 subtypes: α, β, and γ. Each subtype has a different distribution within an organism. Well-known RXR agonists also have no subtype selectivity, except for Net-3IP (an RXRα/β dual agonist) [35]. The NR cofactor assay for RXRs shows that drupanin activated all RXR subtypes to a similar degree, which means that drupanin has no subtype selectivity for RXRs (Fig. 3). It has high selectivity for RXRs relative to RARs, unlike 9-*cis* retinoic acid, which activates RXRs and RARs [26] (Fig. 4A). This indicates that drupanin is a new naturally occurring rexinoid. Although drupanin did not show agonist activity for LXRα and VDR, the results of the NR cofactor assay suggest that it probably possesses PPARα and γ agonist activity. PPAR/RXR heterodimers are

classified into permissive heterodimers, which are not only activated by PPAR or RXR agonists alone, but are also synergistically activated in combination with PPAR and RXR agonists. Therefore, in luciferase reporter assay using pPPREx3-tk-Luc reporter plasmid and pCMX-PPAR expression vector, the activation of reporter plasmid is induced by PPAR or RXR agonist via PPAR/RXR heterodimer Furthermore, the promoter region of pPPREx3-tk-Luc reporter plasmid contains hormone response elements (referred to as DR1) consisting of direct repeats of the core AGGTCA half sites separated by one nucleotide, and it is known to serve as not only PPAR but also RXR response element. This indicates that this reporter plasmid can respond to PPAR/RXR heterodimers and RXR homodimer. Therefore, we used an NR cofactor assay, which is not strictly an NR binding assay, although it can determine the ligand binding to PPARs by measuring the ligand-dependent interaction between PPAR and the CBP coactivator. Drupanin stimulated the interaction between PPARγ and CBP in a dose-dependent manner at relatively low concentrations, where a physiological effect may be expected. However, drupanin also stimulated the interaction between PPARα and CBP at high concentrations of more than 50 μM, which are considered to be physiologically ineffective. Based on these results, we concluded that drupanin is a new type of RXR/PPARγ dual agonist with lower EC_{50} values for RXRs than PPARγ.

PPARγ agonists promote lipid accumulation of adipogenically induced 3T3-L1 cells and ameliorate insulin resistance [36,37]. Conversely, 9-*cis* retinoic acid reportedly inhibits adipogenesis [38]. As the present study revealed that bexarotene also suppressed the adipogenesis of 3T3-L1 cells, the activation of PPARγ/RXR heterodimers by an RXR agonist might well prevent lipid accumulation. However, drupanin accelerated the accumulation of lipid droplets. The reason why drupanin produced an effect different from that of the RXR-specific agonist is due to its inherent PPARγ agonistic activity. Although the behavior of the RXR/PPARγ dual agonist was not obvious, magnolol, a naturally occurring PPARγ/RXRα agonist, has been reported to induce adipogenic differentiation in 3T3-L1 cells [39]. However, Zhang et al. suggested that the K_D values of magnolol for RXRα LBD and PPARγ LBD were 45.7 and 1.67 μM, respectively, which indicated that magnolol mainly activated PPARγ [25]. Therefore, drupanin, a novel RXR/PPARγ agonist with a lower ED_{50} value for RXR than PPARγ, possesses properties different from those of magnolol.

RXR agonists have rarely been detected in natural products. In the present study, we suggested that BGP was a potent RXR agonistic material, and identified drupanin as the active component. RXRs play an important role in the regulation of transcriptional activation related to metabolism, differentiation, and embryogenesis. Furthermore, RXR agonists activate RXR/NR heterodimers synergistically with partner NR agonists. Interestingly, BGP contains artepillin C as a PPARγ agonist, which may act synergistically with drupanin. Thus, our investigation revealed that drupanin is most likely to be an important active compound for clarifying the molecular mechanism underlying the biological activity shown by BGP.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbagen.2014.06.011.

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