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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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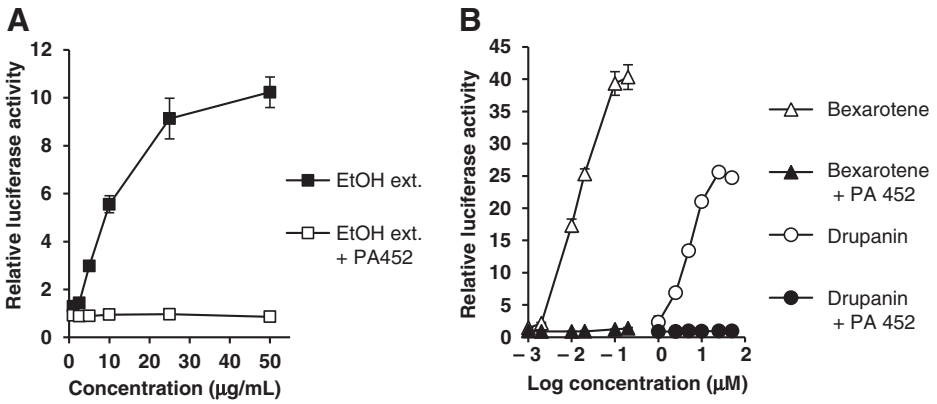

Fig. 1. RXRα agonistic activity of an ethanolic extract of BGP (A) and drupanin (B) in the luciferase reporter assay. HEK293 cells were co-transfected with the RXRα expression vectors and luciferase reporter plasmids together with pCMX β-gal, as described in Section 2.4. At 6 h after transfection, the 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.