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Biologically active components of a Papua New Guinea analgesic and anti-inflammatory lichen preparation

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Abstract

A traditional preparation of *Parmotrema saccatilobum* (Taylor) Hale (Family: Parmeliaceae) is being considered for inclusion into the PNG national drug formulary by the Ministry of Health Taskforce on Traditional Medicines. The lichen preparation is traditionally used in the Milne Bay province of Papua New Guinea for analgesic and anti-inflammatory activities. A hexane extract of *Parmotrema saccatilobum* yielded the principle components atranorin and chloroatranorin. Atranorin and chloroatranorin were tested in a COX-1 and -2 enzyme inhibition assay, which showed that atranorin inhibited COX-1 in a dose dependent manner and suggests partial inhibition by atranorin and chloroatranorin of COX-2 and COX-1, respectively.

Keywords

Parmotrema saccatilobum; lichen; atranorin; chloroatranorin; COX inhibition

1. Introduction

Traditional medicines remain a cost effective and easily accessible health care option in Papua New Guinea [1]. In March 2007, the Papua New Guinea national government formally approved a National Policy on Traditional Medicine promoted by the Ministry of Health [2]. One of the first traditional medicines selected for assessment and development by the Papua New Guinea Traditional Medicines Taskforce, headed by Dr. P. Rai, was a lichen preparation prescribed for superficial joint and muscle pain or inflammation. This traditional preparation is a vegetable oil extract of fresh or dried/stored lichen manufactured by Ms. M. Bate [3]. The traditional preparation calls for 100 grams of lichen to be boiled in approximately 600 mL vegetable oil for 2 to 3 hours after which time the preparation is cooled, and the clear solution decanted for direct topical application [4].

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This work describes the isolation and identification of two principle metabolites, atranorin [5] and chloroatranorin [6], from this traditional medicine preparation. Previously, atranorin and chloroatranorin have been shown to possess antioxidant activity [7,8]. Additional studies on atranorin demonstrated that it effectively inhibits the biosynthesis of leukotriene B₄ in bovine polymorphonuclear leukocytes, which could also lead to an anti-inflammatory effect [9]. While such antioxidant and leukotriene inhibitory activity could conceivably contribute to the anti-inflammatory effects, the structural similarity between these metabolites and known cyclooxygenase inhibitors warranted investigation.

2. Experimental

2.1 Lichen collection

Parmotrema saccatilobum (Taylor) Hale (Family: Parmeliaceae) was collected by Ms. Bate of Alotau, Milne Bay province, Papua New Guinea, with Dr. Rai, University of Papua New Guinea, from the bark of various trees. The lichen was easily separated from its bark substrate to yield a collection visibly free of contamination. Taxonomic information on the lichen species was provided by Dr. Simone Louwhoff of the Royal Botanic Gardens, Melbourne. Voucher samples are held at the Royal Botanic Gardens, Melbourne and at the University of Papua New Guinea, Taurama campus in the laboratory of Professor Rai.

2.2 Extraction and Isolation

Five grams of lichen, *P. saccatilobum*, was extracted with 200 mL hexanes (2 × 24 hr). The hexane extract was dried by rotary evaporation to yield 31.9 mg. A portion (8 mg) of the dried extract was dissolved in DMSO and separated by HPLC using a 10 × 250 mm, phenyl hexyl column. A gradient from 50% CH₃CN/H₂O to 100% CH₃CN over 15 minutes was used. Purified atranorin (3.2 mg) eluted at 11.8 minutes and chloroatranorin (3.8 mg) eluted at 12.8 minutes.

2.3 Cyclooxygenase Inhibition Assay

The ability of atranorin and chloroatranorin to inhibit cyclooxygenase (COX) was tested using the COX (ovine) inhibitor screening assay (#760101) supplied from Cayman Chemical Co., Ann Arbor, MI, USA. Briefly, this kit determines COX activity through quantification of the production of prostaglandin H₂ (PGH₂) via an enzyme immunoassay. Inhibitory compounds were added to a mixture containing enzyme, either COX-1 or COX-2, and the substrate arachidonic acid. Cyclooxygenase converts arachidonic acid to PGH₂. After incubation, the reaction mixture was added to a 96 well plate coated with a prostaglandin antibody and incubated for 18 hours. The plate was developed and read on a plate reader at 405 nm to quantify the production of PGH₂. Percent inhibition was calculated based upon a standard curve. For this assay, atranorin and chloroatranorin, dissolved and diluted in DMSO, were tested alongside acetylsalicylic acid (positive control) for inhibitory activity against COX-1 and COX-2.

3. Results and discussion

Purification of compounds directly from the vegetable oil preparation was unsuccessful due to the high abundance of lipids. In an attempt to identify components that might be present in the vegetable oil extract, the lichen was extracted with hexane and the extract separated by HPLC. Although hexane extraction does not completely mimic the original preparation, it should provide a representation of chemistry extracted into vegetable oil. NMR analysis of the crude hexane extract was performed at 400 MHz using a Varian Mercury spectrometer in CDCl₃/CD₃OD and indicated that the extract was comprised of two major components (Fig. 1), accounting for approximately 95% of the extract weight. Further NMR (*vide infra*) and MS

analysis identified these components as the known lichen metabolites atranorin and chloroatranorin.

NMR analyses included ^1H , ^{13}C , gHSQC, and gHMBC experiments. NMR spectra for the pure compounds were recorded on a Varian INOVA at 500 MHz for ^1H and 125 MHz for ^{13}C using vendor supplied pulse sequences. Residual solvent (CDCl_3) signals were used as reference (δ_{H} , 7.24; δ_{C} , 77.0 ppm). NMR data for atranorin matched those reported by de Carvalho, *et al.* [5]. NMR data for chloroatranorin matched those reported by Huneck and Yoshimura [6]. Accurate mass measurements were performed on a Micromass Q-tof Micro using positive electrospray with leucine enkephalin as a reference mass at m/z 55-.2771. For atranorin, an ion was observed for $[\text{M}+\text{Na}]^+$ at m/z 397.0903 (calc'd for $\text{C}_{19}\text{H}_{18}\text{O}_8\text{Na}$, 397.0899). For chloroatranorin, an ion was observed for $[\text{M}+\text{Na}]^+$ at m/z 431.0507 (calc'd for $\text{C}_{19}\text{H}_{17}\text{O}_8^{35}\text{ClNa}$, 431.0510). After identification of the atranorins, UV studies were undertaken on a Hewlett Packard 8452A diode array spectrophotometer to confirm the presence of the metabolites in the traditional medicine preparation. Vegetable oil has a weak UV absorbance, UV spectra maximum of the traditional preparation that corresponded to atranorin and chloroatranorin was observed. The overlay of UV spectra from the vegetable oil, atranorin, and chloroatranorin indicating overlapping maxima are in Figure 2. While it is not proven that the spectrum of the traditional preparation is due to atranorin and chloroatranorin, the characteristic spectrum generated by simple UV analysis could nevertheless be used for batch to batch standardization of traditional preparations.

Inflammation, pain and pyresis are often partially attributable to elevated levels of prostaglandins. Systemic and topical nonsteroidal anti-inflammatory drugs are a mainstay of over-the-counter analgesic preparations and work by inhibiting the COX enzymes, also called Prostaglandin H Synthase [10]. Two human forms of cyclooxygenase have been described, COX-1 and COX-2. COX-1 is constitutively expressed in a variety of cell types and is involved in normal cellular homeostasis. COX-2 is also constitutively expressed in some tissues but is often induced during the inflammatory process [11].

Structural similarity between atranorin and chloroatranorin and known cyclooxygenase inhibitors prompted investigation into the ability of these compounds to inhibit COX-1 and COX-2. The ability of atranorin and chloroatranorin to inhibit these enzymes was determined using a commercially available kit that quantifies prostaglandin production. As shown in Fig. 3, atranorin inhibited COX-1 in a dose dependent manner with approximately 50% of enzyme activity inhibited at 17 $\mu\text{g/mL}$ (~45 μM). Approximately 40% inhibition of COX-2 and COX-1 enzyme activity was observed for atranorin and chloroatranorin, respectively, at all concentrations ranging between 17 $\mu\text{g/mL}$ and 0.17 $\mu\text{g/mL}$ (45 μM and 0.45 μM), data not shown. While dose dependent relationships were not observed, these results suggest partial inhibition. Chloroatranorin did not exhibit any activity in the COX-2 assay. Acetylsalicylic acid (positive control) at a final concentration of 50 μM exhibited 59% and 42 % inhibition of COX-1 and COX-2, respectively.

Data presented here suggest that part of the analgesic effect obtained with this traditional medicine may be due to inhibition of the cyclooxygenase enzymes by atranorin and chloroatranorin. This work lays the foundation for future assessment and development of this lichen traditional medicine. Additional anti-inflammatory experiments should provide a greater understanding to the mode of action of these compounds. Also, while atranorin and chloroatranorin were determined to be the major components of the hexane extract, further analysis of the traditional medicine preparation should be conducted to identify any other bioactive components.

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Figure 1.
 ^1H spectrum of crude hexane extract in $\text{CD}_3\text{OD}/\text{CDCl}_3$ at 400 MHz.

Figure 2.

UV spectra showing overlap between atranorins and the traditional preparation.

UV spectra of the COX-inhibitory components isolated from a Papua New Guinean analgesic and anti-inflammatory lichen preparation.

Figure 3.

Dose dependent inhibition of COX-1 by atranorin. The ability of atranorin to inhibit the COX-1 enzyme was assessed by monitoring the production of prostaglandin in the presence of increasing concentrations of atranorin. Data is representative of experiments repeated three times and is graphed as the average of duplicate determinations.