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Key cytokines of adaptive immunity are differentially induced in rainbow trout kidney by a group of structurally related geranyl aromatic derivatives

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Filifolinone is a semi-synthetic terpenoid derivative obtained from *Heliotropium filifolium* that increases the expression level of pro-inflammatory and anti-inflammatory cytokines in kidney cells of salmon. Because cytokines are produced in response to a foreign organism and by distinct other signals modulating immune responses, we further studied the potential immunomodulatory effects of a group of structural related terpenoid derivatives from *H. filifolium* on salmonids to determine the relationship between the chemical structure of the derivatives and their ability to modify cytokine expression and the lymphoid content. The resin and four 3H-spiro 1-benzofuran-2,1′-cyclohexane derivatives were tested *in vivo* in rainbow trout (*Oncorhynchus mykiss*) by quantifying the transcript levels of antiviral and T helper-type cytokines and T and B cells in the kidney. Three of the four terpenoids differ only in the C-7′substituent of the cyclohexane and the presence of the ketone group at this position in Filifolinone appeared responsible of an important up-regulation of IFN-α1, IFN-γ, IL-4/13A and IL-17D in the kidney of the treated trout. In addition, the absence of a methoxy group in carbon 7 of the benzene ring, found in all compounds but not in Folifolinoic acid, produced a significant reduction of IFN-γ, IL-12 and IL-4/13A transcripts. B cells were not affected by the compound treatment but Filifolinoic acid and the resin induced a significant reduction of T cells. Altogether, results showed that immunomodulating responses observed in the trout by effect of 3H-spiro 1-benzofuran-2,1′-cyclohexane derivatives is related to the presence of the ketone group in the carbon 7′ and the methoxy group in carbon 7 of the benzene ring, being Filifolinone the most active immunostimulatory compound identified.

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1. Introduction

During the last decades, search for natural immunostimulant compounds has increased considerably; two examples of success are the MF59, which is an immunologic adjuvant that uses *squalene* as the oil-in-water emulsion [1] and the carotenoids, which are used as antioxidants. Compounds increasing cytokine gene expression can be effective immunostimulants because cytokines are key molecules regulating immune system. In fact cytokines can activate inflammatory and anti-inflammatory signals, regulate antigen presenting cells (APC) and also play a role on the effector functions and homing properties of T and B cells, including differentiation into memory cells [2]. Many cytokines of the network have been also described in fish [3]. Although knowledge has arisen mostly from transcriptome and genome analyses, some functional data generally using recombinant fish cytokines showed they have similar roles to their homologues in mammals. For example, fish IFN-γ induces potent antiviral activity against salmonid alphavirus 3 and significantly stimulated gene expression of IFN-γ-inducible

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protein 10, MHC class II β-chain and STAT1 demonstrating a functional IFN-γ homologue in fish [4,5].

Plants are a great source of bioactive chemicals and thereafter the biological properties of active compounds have been extensively studied. Among these compounds, terpenes and flavonoids have been shown to have antioxidant, antiviral and antibacterial properties. In this regard, we have studied bioactive compounds of plants of the genus *Heliotropium* section *Cochranea*, which produce resinous exudates as a defense mechanism against the adverse environmental conditions under which the plant grows [6]. This resin contains mainly flavonoids along with aromatic geranyl derivatives in minor quantities [7–10]. The specie *Heliotropium filifolium* grows in arid areas with extreme environmental conditions such as low temperatures, lack of water, wounding, low nutrients, and presence of pathogen attacks whereas produce a resin that cover the leaves and stems as a first stage of protection against predators. Protective effects of the resin is due to the presence of secondary metabolites, such as Filifolinol, the first example of a spiro benzohydrofuranyl terpene, and the ester derivative filifolinyl senecionate, which exhibit antimicrobial and antioxidant activities [7,11–16].

Most recently we demonstrated that the terpenoid semi-synthetic 3H-spiro [1-benzofuran-2,1′-ciclohexane] derivative, called Filifolinone [11] has immunostimulant activity as it increases the expression level of pro-inflammatory and anti-inflammatory cytokines in SHK-1 cells and in kidney cells when Atlantic salmon (*Salmo salar*) were injected with Filifolinone [17]. Interestingly, Filifolinone induces transcript increase of cytokines of the adaptive immunity IFN-γ, IL-12, TGF-β1 and IL- 10 and of the anti-viral cytokine IFN-α1 in the kidneys of treated fish [15]. Filifolinone showed also immunomodulatory effects on mammals by promoting maturation of dendritic mouse cells [18]. Because these terpenoid molecules stimulating cytokine gene expression can be highly useful in immunotherapy and vaccine formulation for fish, here we seek to further study the potential immunomodulatory effects of structural related terpenoid derivatives from *H. filifolium* on salmonids to determine the relationship between the chemical structure of the derivatives and their ability to modify cytokine expression and the lymphoid content. Four of these 3H-spiro 1-benzofuran-2,1′-cyclohexane derivatives with discrete differences in their structure were purified or chemically derivatized in order to understand how such differences can influence the immunomodulatory effects. Compounds studied were Filifolinone, which is obtained by oxidation of Filifolinol [9], the main compound isolated from resinous exudates of *H. filifolium* and two other derivatives, Filifolinyl senecionate [13] and Filifolinoic acid [14]. Immunomodulatory effects were studied *in vivo* on rainbow trout (*Oncorhynchus mykiss*) by quantifying the expression levels of cytokines and the percentage of lymphoid cells in kidneys.

2. Materials and methods

2.1. Extraction and isolation of the natural compounds and resin

H. filifolium (Miers) Reiche samples were collected in Carrizal Bajo in the north of Chile, III region during the flowering season. A voucher specimen was deposited in the Herbarium of the Faculty of Biological Science of the Catholic University of Chile (ST-2214 SSUC). The resinous exudate was extracted by immersion of the fresh plant material (900 g) in dichloromethane (CH_2Cl_2) for 30 s at room temperature and was concentrated to a sticky residue (220 g). Part of the extract (25 g) was purified by CC (silica gel) using hexane-EtOAc step gradient. Filifolinol was obtained as a white solid and purified by recrystallization of hexane being obtained 7.1 g of pure compound. The compound was identified by comparison with the NMR^{1}H spectrum of the standard sample [5]. Filifolinyl senecionate was purified by preparative column using hexane-EtOAc (4:1) to afford 360 mg as color less oil. Its structure was identified by NMR^{1}H, NMR^{13}C and HRMS as indicated previously [13].

2.2. Semi-synthesis of compounds

Filifolinone was obtained from Filifolinol. Hence, 230 mg of Filifolinol were dissolved in 5 mL of anhydrous pyridine and 240 mg of CrO_3 were added. The mixture was left at room temperature for 18 h and then poured on 5 mL of methanol. Water was then added and ether extractions were performed. A solid compound was obtained. The compound was purified by column chromatography using benzene-ethyl acetate (95:5) and recrystallized of methanol being obtained 190 mg of pure white solid [11]. Filifolinoic acid was obtained from Filifolinol by basic hydrolysis with NaOH and purified by recrystallization [14]. Spectroscopic analyses (NMR^{1}H, NMR^{13}C, HRMS) were done to determine the structure of the purified compound [11]. For Filifolinoic acid synthesis, 1.36 g of Filifolinol was dissolved in distilled water (10 mL) and methanol (10 mL) and 1.0 g of NaOH was added. The mixture was left at room temperature for 24 h. Subsequently, HCl was added until pH 1. The white solid product was obtained. This compound was purified by column chromatography (silica gel) using a hexane-ethyl acetate step gradient to afford 625 mg of the pure compound. The structure was identified by NMR^{1}H, NMR^{13}C and HRMS [14].

2.3. Cytotoxicity assay

Cytotoxic effects of the compounds were tested on leukocytes isolated from kidneys of rainbow trout. Briefly, the cells were incubated in Leibovitz 15 medium (L15; Gibco, Invitrogen, Carlsbad, CA, USA), supplemented with 10% fetal calf serum (FCS; Hyclone, Thermo Fisher Scientific, Logan Utah, USA), 4 mM L-glutamine (Gibco), 50 μM 2-mercapthoetanol (2-ME; Gibco) and 50 μg/mL gentamicin (US Biological, Swampscott, MA, USA) for 12 h at 15 °C with 0.5, 20 and 100 μg/mL of each terpene derivative solubilized using dimethyl sulfoxide (DMSO; final concentration 0.2%). Viability of the cells after incubations was assayed by staining with 2 μL with 1 mg/mL of propidium iodide. Analyses were done by flow cytometry in a FACSCanto II Cytometer; at least 5000 events were recorded for each sample. FACSDiva software was used for the analysis. For positive control, cells were incubated with 30% ethanol and as negative control cells were treated with medium containing 0.2% of DMSO, which is used to solubilize the compounds.

2.4. Fish and treatments

Rainbow trout (*O. mykiss*) were obtained from a local fish farm and maintained in tanks with a freshwater system at a biomass of 10–12 kg/m^3, at 12–16 °C and with continuous support of air. Fish were fed with commercial pellets once a day and acclimated for 2 weeks previous to treatment.

For the study, 64 trout of approximately 50 g were selected at random and divided into 8 groups. Fish were injected intramuscularly (i.m.) with the compounds: tests groups received 100 μg of either Filifolinol, Filifolinone, Filifolinyl senecionate, Filifolinoic acid or Resine; control groups received 100 μL of either sterile 0.9% NaCl solution which was the vehicle of injection, 8% DMSO and one group received no treatment. Fish were sacrificed 48 h post-injection with benzocaine (Veterquímica). From each specimen, whole kidneys were extracted and one half of the organ was stored at 80 °C for RT-PCR analyses and the other half was processed for flow cytometry analyses.

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