Diayangambin Exerts Immunosuppressive and Anti-Inflammatory Effects *in vitro* and *in vivo*

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Abstract

In this study, the furofuran lignan (+)-diayangambin [tetrahydro-1,4-bis(3,4,5-trimethoxyphenyl)-(1*R*)-1α,3aβ,4α,6aβ-1*H*,3*H*-furo [3,4-*c*]furan] was evaluated *in vitro* and *in vivo* for its immunomodulatory and anti-inflammatory efficacy. Human mononuclear cell proliferation was inhibited by diayangambin with an IC_{50} value of 1.5 (0.5 – 2.8) μM. In addition, the compound reduced for 40.8 % prostaglandin E_2 generation in stimulated RAW 264.7 macrophage cell line at 10 μM. *In vivo*, a clear reduction of ear swelling was observed when diayangambin (40 mg/kg) was administered orally to 2,4-dinitrofluorobenzene-treated mice. The inhibition of swelling was associated with a reduction of leukocyte infiltration determined as myeloperoxidase activity. In the carrageenan mouse paw edema model, diayangambin significantly suppressed inflamed paw volume and prostaglandin E_2 levels. Our findings indicate the potential interest of diayangambin in the treatment of immune and inflammatory responses.

Activated T lymphocytes have been reported to play an important role in the development and pathogenesis of inflammatory, allergic and autoimmune diseases. Their activation involves cell proliferation through multiple intracellular signaling pathways, with interleukin-2 being the main lymphokine involved [1]. Macrophages also participate in host defense, immunity and inflammatory responses, where they produce a wide array of mediators such as cytokines, oxygen and nitrogen species and high levels of prostaglandin E_2 (PGE_2) upon induction of cyclo-oxygenase-2 (COX-2) [2].

Lignans are natural products with a variety of biological activities [3]. According to the literature, many of them present immunomodulatory, anti-allergic, and anti-inflammatory activities by inhibition of T-cell proliferation or inhibiting several pro-inflammatory mediators such as cytokines, eicosanoids, and the platelet activating factor [4], [5], [6], [7]. Recently, the furofuran lignan (+)–diayangambin (Fig. **1**) and other lignan derivatives

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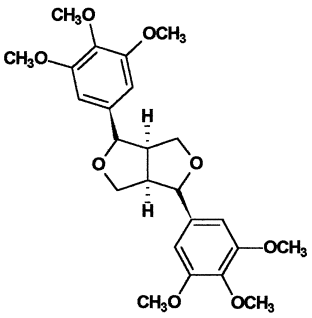

Fig. **1** Structure of diayangambin.

were isolated from the leaves of *Piper fimbriulatum* C.DC. [8]. This species, growing in Panama, belongs to a large genus containing ca. 700 species, many of them used in South American folk medicine for several purposes including inflammatory processes [9,10,11]. In the present work, we investigated the immunomodulatory and anti-inflammatory activity of diayangambin *in vitro* and *in vivo*.

Co-incubation of human lymphocytes with diayangambin inhibited the phytohemoaglutinin(PHA)-induced ^3H-thymidine incorporation into human lymphocytes in a concentration-dependent manner (Fig. **2**), with an IC_{50} value of 1.5 (0.5 – 2.8) μM. Diayangambin was more potent that the reference compound azathioprine (IC_{50}: 11.9 (4.5 – 19.9) μM) but showed lower activity than dexamethasone (IC_{50}: 6.0 (3.0 – 16.1) nM). Inhibition of lymphocyte proliferation was not due to cytotoxic effects of diayangambin, since viability, assessed by the lactate dehydrogenase (LDH) assay, was not decreased after co-incubation of cells with this compound.

Stimulation of the RAW 264.7 murine macrophage cell line with bacterial lipopolysaccharide (LPS) causes expression of COX-2, with the consequent generation of large quantities of PGE_2. Twenty-four hours-LPS-stimulated RAW 264.7 macrophages produced 34.8 ± 3.7 ng/ml of PGE_2 with respect to 5.2 ± 0.2 ng/ml of PGE_2 in untreated cells. The production of this eicosanoid was significantly reduced by diayagambin at 10 μM (40.8%). As expected, NS398 (COX-2 inhibitor) showed a high inhibitory effect (95%) at the same concentration. None of these compounds affected cellular viability, as assessed by mitochondrial reduction of 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan.

CD4$^+$Th1 and CD8$^+$ type 1 cytotoxic T cells are crucial effector cells in the delayed-type hypersensitivity (DTH) response to 2,4-dinitrofluorobenzene (DNFB) in mice [12]. We selected this animal model to assess the possible immunosupressive effect of diayangambin *in vivo* (Fig. **3**). Administration of diayangambin (40 mg/kg *p.o.*) to mice for three consecutive days before DNFB challenge, significantly inhibited the ear swelling after 24 h (42% inhibition). In addition, myeloperoxidase activity, determined in ear homogenates as leukocyte infiltration index, was reduced (61% inhibition). The reference compound dexamethasone (3 mg/kg *p.o.*) also suppressed both parameters.

In the mouse carrageenan paw oedema model, diayangambin (40 mg/kg *p.o.*) significantly inhibited the formation of oedema 3 h and 5 h after induction of inflammation (Fig. **4**). The greatest effect was observed at 5h, with an inhibition of 48.8%. Indomethacin (10 mg/kg) showed a significant reduction of the swelling (46%) after the same time. The last evaluation of oedema (5 h) was followed by killing of the animals and determination of the PGE_2 content in paw homogenates. The results indicate that diayangambin significantly reduced PGE_2 levels in inflamed paws (30.9%). As expected, indomethacin strongly inhibited the levels of this eicosanoid (98%).

In conclusion, the present data show that diayangambin possesses *in vitro* modulatory activity on lymphocyte proliferation and PGE_2 generation in macrophages. These results are consistent with the anti-inflammatory profile shown *in vivo* in DTH response and mouse paw oedema models. The ability of diayangambin to modulate lymphocyte and macrophage responses may be useful in the treatment of immune and inflammatory responses.

Materials and Methods

(+)-Diayangambin ($[\alpha]_D$ +283.4°, $CHCl_3$) was isolated from the leaves of *Piper fimbriulatum* C.DC. following known procedures [8], [13], [14]. Plant material identification was established by Prof. Mireya Correa, Director of the Herbarium of the University of Panama where a voucher specimen is preserved (Florpan

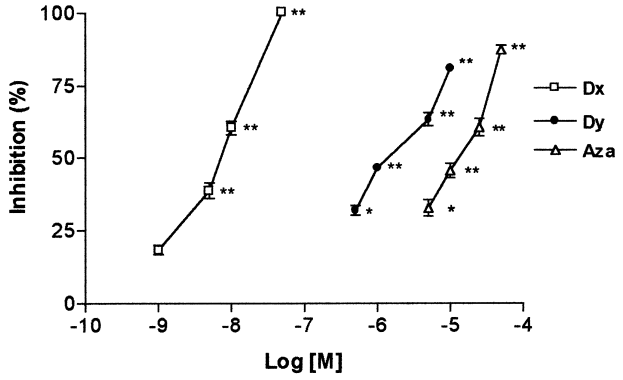

Fig. **2** Antiproliferative effects of diayangambin (Dy), azathioprine (Aza) and dexamethasone (Dx) on PHA-stimulated human lymphocytes. Control value was 14476 ± 374 cpm. Data are expressed as % of inhibition (mean ± S.E.M.) of at least three different experiments. * $P < 0.05$, ** $P < 0.01$

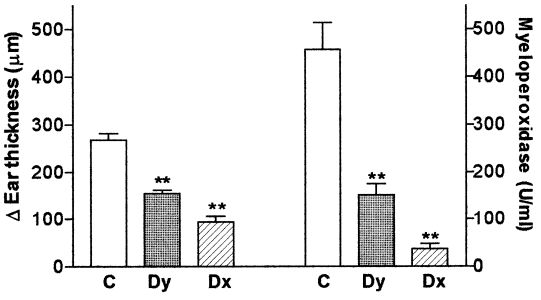

Fig. **3** Effect of diayangambin (40 mg/kg *p.o.*) and dexamethasone (3 mg/kg *p.o.*) on DNFB-delayed hypersensitivity. Data correspond to the increase in ear thickness and myeloperoxidase levels in ear homogenates. C = vehicle; Dy = diayangambin; Dx = dexamethasone. Data represent means ± S.E.M. (n = 8 – 12 animals). ** $P < 0.01$.

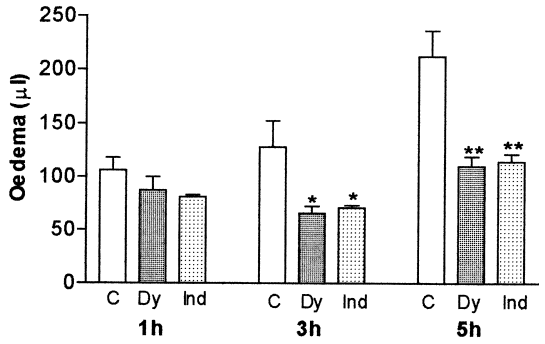

Fig. **4** Effect of diayangambin (40 mg/kg *p.o.*) and indomethacin (10 mg/kg *p.o.*) on carrageenan-induced mouse paw oedema 1, 3, and 5 h after induction of inflammation. C = vehicle; Dy = diayangambin; Ind = indomethacin. Data represent means ± S.E.M. (n = 8–12 animals). * $P < 0.05$, ** $P < 0.01$.

2790). [5,6,8,11,12,14,15(n)-^3H]-PGE_2 and [methyl-^3H]-thymidine were from Amersham Iberica (Madrid, Spain). The rest of reagents were from Sigma Chem. (St. Louis, MO, USA).

Peripheral blood mononuclear cells were isolated from buffy coats obtained from healthy blood donors by Ficoll-Paque density gradient centrifugation [15]. The mononuclear cells interphase was carefully aspirated and washed twice in saline. Then, cells were resuspended in RPMI-1640 media supplemented with 2 mM L-glutamine, 15 mM $NaHCO_3$, 10 mM HEPES, 100 I.U./ml penicillin and 100 µg/ml streptomycin with 10% heat-inactivated foetal bovine serum (FBS). The cell suspension was placed in culture Petri dishes and after incubation for 1 hour non-adherent cells were collected. They were cultured in 96-well tissue culture plates in a volume of 200 µl/well (1×10^6cells/ml) in the presence of 12 µg/ml of PHA. Compounds or vehicle (1% methanol) were added before mitogen stimulus. The cells were cultured for 72 h at 37 °C in a humidified atmosphere containing 5% CO_2 and then pulsed for 18 h with [methyl-^3H]-thymidine (0.08 µCi/well). Cells were harvested and thymidine incorporation measured with a Microbeta trilux counter (Wallac, Turku, Finland). The cytotoxicity of diayangambin was assessed using the LDH release assay [16].

Murine macrophage cell line RAW 264.7 (Cell Collection, Department of Animal Culture, C.S.I.C., Madrid, Spain) was cultured in DMEM medium containing 2 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin and 10% FBS. Cells were resuspended at a concentration of 2×10^6/ml and co-incubated in 96-well culture plate (200 µl) with 1 µg/ml of *Escherichia coli* [serotype 0111:B4] LPS at 37 °C for 20 h in the presence of test compounds or vehicle. PGE_2 levels were assayed in culture supernatants by radioimmunoassay. The mitochondrial-dependent reduction of MTT to formazan was used to assess the possible cytotoxic effect of diayangambin [17].

In DNFB-induced DTH, mice were topically sensitized with 0.2% (v/v) DNFB solution (20 µl) in acetone onto the shaved abdomen on days 0 and 1(total 80 µg). Five days after the initial sensitization, mice were exposed to 0.2% DNFB (10 µl) on both sides of the right and left ears. Diayangambin (40 mg/kg) and dexamethasone (2 mg/kg) were orally administered in propylene glycol/distilled water (50 : 50, v/v) to mice for three consecutive days (72, 48 and 24 h) before DNFB challenge. Ear thickness was measured 24 h after challenge using a digital micrometer (Mitutoyo, Japan). The oedema was calculated for each ear as the difference in thickness before and 24 h after challenge. Animals were killed by cervical dislocation and ear sections were homogenized to measure myeloperoxidase activity in supernatants [18].

Mouse paw oedema was induced by injection of carrageenan lambda type IV (0.05 ml; 3% w/v in saline) into the subplantar area of the right hind paws of groups of six animals. Diayangambin and indomethacin were orally administered 1 h before injection of carrageenan. The volumes of injected and contralateral paws were measured at 1, 3, and 5 h after induction of oedema by using a plethysmometer. Finally, mice were sacrificed by cervical dislocation and hind paws were homogenized in saline to determine PGE_2 levels by radioimmunoassay [19].

Statistical analysis: The results are presented as mean ± S.E.M. IC_{50} values were calculated from at least four significant concentrations (n = 6). When appropriate, 95% confidence limits were calculated. The level of statistical significance was determined by analysis of variance followed by Dunnett's *t*-test for multiple comparisons.

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