Immunomodulatory Activity of Sulfonamide Chalcone Compounds in Mice Infected with Filarial Parasite, *Brugia malayi*

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Abstract Diethyl carbamazine (DEC) is being used as a sole drug to treat the lymphatic filariasis, although encountered with many limitations. Importantly, DEC works with putative host immunomodulating activities without any direct antifilarial effect. This study aimed to assess the possible modulatory effect on host immune system by sulfonamide chalcone compound, having direct antifilarial activity. The immunomodulatory activity of DEC and/or chalcone compound, 4t on mice peritoneal exudate cells (PECs) was analyzed initially in vitro. This was followed by the study of in vivo effect of these test agents in the parasitaemic BALB/c mice induced by *Brugia malayi* microfilariae. Cytokine profile and iNOS induction were measured from PECs of mice. 4t compound showed anti-inflammatory activity in vivo in contrast to DEC. Further 4t was found to increase anti-inflammatory and regulatory cytokines, IL-10 and TGF- β gene expression with down regulation of pro-inflammatory cytokines TNF- α and IFN- γ and iNOS in mice PECs in vitro. In conclusion, chalcones having direct antifilarial effect also upsurges anti-inflammatory host immune response. Therefore, the results might be envisaged as 4t to be a better alternative to DEC in the chronic case of lymphatic filariasis.

Keywords Immunomodulation · *Brugia malayi* · Sulfonamide chalcones · Anti-inflammatory activity

Introduction

Lymphatic filariasis is a mosquito borne disease and caused by parasitic nematodes, *Wuchereria bancrofti*, *Brugia malayi* and *B. timori*. A total population of 947 million people in 54 endemic countries and territories are at risk for lymphatic filariasis [1]. Moreover, 36 million people are suffering with chronic manifestations. Presently, diethyl carbamazine (DEC) has been widely used antifilarial drug in lymphatic filariasis treatment.

Host-microbe interaction plays significant role in elimination of parasite from the host. Being the classical defense stratagem against microbial pathogen, host immune response elicit pro-inflammatory impact at both cellular and molecular level. The major modus operandi of DEC also exhibits indirect antifilarial mode that activates macrophages and other innate immune cells to sequester and kill microfilarial (Mf) stage of parasite. However, such immune cell mediated pro-inflammatory reaction exposes the host to the untoward brunt of oxidative stress which may result into side effects in the host, contributing to the poor patient compliance.

Many researchers have reported the immunomodulatory activity of chalcone derivatives [2]. Though many chalcone derivatives induce pro-inflammatory immune response, others have been shown to exhibit anti-inflammatory properties, having beneficial role in various inflammatory diseases [2, 3]. Earlier, we have reported a series of sulfonamide chalcone derivatives having direct microfilaricidal activity under in vitro condition in the absence of any host immune cells [4]. Among them, in the present study,

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4t compound was selected as a representative owing to its lower IC₁₀₀ value and good therapeutic window, for elucidation of possible immunomodulatory effect in host.

Materials and Methods

In Vitro Effect on Mouse PECs

Sulfonamide chalcone derivative, 4t, was synthesized and characterized as described earlier [4]. Murine peritoneal exudate cells (PECs) were harvested by the method previously described [5]. Normal BALB/c mice were injected with 2 ml of 1% thioglycollate medium into the peritoneal cavity and on the fourth day PECs were harvested by lavage with 10 ml of ice cold and sterile PBS (0.05 M; pH 7.2) containing 20 mM ethylenediaminetetraacetate (EDTA). The cells were subjected to washing in RPMI 1640 medium supplemented with antibiotics.

Cells were enumerated and plated in triplicates (2×10^5 cells/100 μ l/well) in 96 well flat-bottom cell culture plates. After 4 h of incubation, non-adherent cells were washed off. Adherent cells were then stimulated with Lipopolysaccharide from *Escherichia coli* (LPS; 10 μ g/100 μ l/well) in the presence or absence of 4t and/or DEC with comparable concentration (10 μ g/100 μ l/well). PECs incubated with medium alone represented as control. After 24 h of incubation (5% CO₂ at 37 °C), cells were pelleted by centrifugation and stored in 1 ml of Trizol reagent at – 80 °C until used to estimate the mRNA levels of cytokines using real-time PCR analysis. Cell culture supernatants were separately collected to measure the nitrite levels using Griess reagent.

NO Estimation by Spectrophotometric Method

Griess reagent (100 μ l) was added to 100 μ l of culture supernatants in 96-well flat bottom immuno-plate and incubated for 10 min at room temperature. The development of color was measured by reading the plate at 540 nm. Suitable control without culture supernatant was also included in the assay as reagent blank. Varying concentrations of sodium nitrite (NaNO₂) ranging from 50 to 400 nM was used to plot a standard curve and concentration of nitrite in test samples was calculated [6].

In Vivo Effect on BALB/c Mice

Female BALB/c mice (6–8 weeks old), bred and maintained in the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India registered animal house facility, were used in this study. The mice were housed and fed under

standard laboratory conditions. All experiments were approved by the Institutional Animal Ethics Committee.

Mice were divided into different groups (n = 6 in each group) viz., Control mice (normal mice treated with 0.1% Tween 80 vehicle), DEC group (normal mice treated with DEC), 4t group (normal mice treated with 4t), Mf group (parasitaemic mice), Mf-DEC group (parasitaemic mice treated with DEC), Mf-4t group (parasitaemic mice treated with 4t) and Mf-4t-DEC group (parasitaemic mice treated with 4t and DEC). Parasitaemia was induced by injecting 400,000 Mf in a volume of 200 μ l intravenously into mice. Systemic parasitaemia was allowed to equilibrate for 24 h. Establishment of parasitaemia was confirmed by tail bleeding of heparinised blood samples and checking the presence of Mf [7].

BALB/c mice were injected intraperitoneally with 10 mg/kg DEC in endotoxin-free water and/or 100 mg/kg 4t in 0.1% Tween 80 or 100 μ l of 0.1% Tween 80 vehicle only. Dose of DEC was determined as per previous reported work [7]; while, to get the maximum effect of 4t drug on host immune system to explore its immunomodulatory activity, 4t drug was used at highest concentration (100 mg/kg) as reported earlier [8]. After 30 min, PECs were collected by lavage in sterile phosphate buffered saline (0.05 M; pH 7.2) containing 20 mM ethylenediaminetetraacetate (EDTA). The cells were pelleted and stored in 1 ml of Trizol reagent at – 80 °C until used to estimate the mRNA levels of cytokines.

Assessment of Relative mRNA Expression of Cytokines in PECs

Total RNA was isolated from the PECs of the mice of different groups and reverse transcribed to cDNA by using a TRIZol reagent and High Capacity cDNA Reverse Transcription Kit (Invitrogen, Mumbai) according to the manufacturer's instructions. Real time PCR was performed on a StepOne Plus machine (Applied Biosystems, Mumbai) using TaqMan gene expression assays (Applied Biosystems, Mumbai) for cytokines IFN- γ , TNF- α , IL-10, TGF- β and iNOS. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous control. Relative mRNA expression by each of the various study groups of mice (against the corresponding mRNA expression by normal control mice as baseline) was analyzed using StepOne analysis software 2.2.2 (Applied Biosystems, Mumbai).

Statistical Analysis

Statistical analyses were carried out using software SPSS 21.0 (IBM, Armonk, NY, USA). Continuous variables were expressed as mean $\pm$ standard deviation (SD). The

variables were checked for normality assumptions. Normally distributed data were analyzed using suitable test for comparison of means. Data, which is not normally distributed, were analyzed using suitable nonparametric test and represented as median and range. P values ≤ 0.05 were considered as statistically significant.

Results

In Vitro Effect on Mouse PECs

LPS, a known endotoxin, was found to up-regulate IFN- γ (median 0.861; range 0.72–0.92) and TNF- α (median 0.346; range 0.33–0.35) cytokine gene expression with increased iNOS expression (median 17.02; range 16.96–18.2) and down-regulate anti-inflammatory and regulatory cytokines i.e. IL-10 (median 0.085; range 0.075–0.098) and TGF- β (median 0.09; range 0.08–0.098) gene expression. DEC, a known immunomodulatory and antifilarial drug, also showed enhanced pro-inflammatory cytokine expression in vitro. However, stimulation with LPS along with 4t drug, led to decrease in inflammatory and pro-inflammatory IFN- γ (undetected), TNF- α (median 0.051; range 0.046–0.057) and iNOS (median 0.12; range 0.10–0.29) genes expression and increase in IL-10 gene

expression (median 0.39; range 0.34–0.48) in PECs (Fig. 1).

Similarly, as shown in Fig. 2, there was significant increase in NO level in LPS treated PECs, which was further enhanced in PECs co-stimulated with DEC. Whereas, 4t significantly reduced LPS or LPS + DEC induced NO generation in PECs.

In Vivo Effect on BALB/c Mice

As shown in Fig. 3a, significant increase in iNOS gene expression was observed in PECs of parasitaemic BALB/c mice after DEC treatment (median 326.74; range 324.65–404.55). Whereas, 4t drug was found to decrease expression of iNOS (undetected) and TNF- α (median 10.29; range 4.69–13.67) gene in PECs of parasitaemic BALB/c mice compared to untreated parasitaemic mice considered as control. We also found definitive reduction in TNF- α gene expression in PECs of parasitaemic BALB/c mice due to co-treatment with DEC and 4t (median 28.03; range 14.89–75.31), which was elevated by administration of DEC alone (median 97.74; range 45.52–231.92); although the difference was not found to be statistically significant (Fig. 3b).

In case of TGF- β gene expression, up-regulation was found in 4t and/or DEC administered microfilaraemic mice

Fig. 1 Effect of LPS or 4t or DEC (single or in combination) on gene expression of **a** anti-inflammatory cytokines **b** pro-inflammatory cytokines and **c** iNOS in PECs of BALB/c mice in in vitro. Results

were analyzed by Kruskal–Wallis test and expressed as median. * $p < 0.001$, ** $p < 0.05$ compared to LPS treated PECs. UD undetected

Fig. 2 Effect of LPS or 4t or DEC (single or in combination) on NO generation in PECs of BALB/c mice in vitro. Results were analyzed by one way ANOVA followed by Tukey's post hoc test and expressed as mean $\pm$ SD. * $p < 0.05$

compared to untreated mice, although there was no significant difference (Fig. 3c).

Discussion

Host-microbe interaction is an important and active area of research. As a general mechanism of antimicrobial response, host immune response upsurges during any infection. Earlier work from our laboratory showed involvement of oxidative stress in active filarial infection

[9]. As mentioned before, DEC exploits antifilarial effect by activating innate immune cells and arachidonic acid expression in both parasites as well as host cells [10]. It modulates and activates macrophages to induce pro-inflammatory immune response with release of NO, which in turn cause killing of Mf [7, 11]. There is report of reduced effect of DEC on the parasite in the microfilaraemic *iNOS*^{-/-} BALB/c mice [7].

DEC induced oxidative and/or nitrosative burst leads to development of local pro-inflammatory microenvironment for host-parasite interaction and might be contemplated to be associated with untoward hyperactivity and non-compliance in patients. In contrast to DEC which is unable to deliver any direct antifilarial effect, the chalcone compound 4t used in present study showed direct therapeutic potential in vitro [4]. Hence, this chalcone may not be expected to be dependent on enhancement of inflammatory response in the host like DEC as its major rationale of action. However, certain earlier study provides evidence that chalcones possess immunomodulatory potential [2]. Therefore, in present study, an exploration of immunomodulatory effect of sulfonamide chalcone compound was considered which could be exploited as a possible adjunct to the drug development strategy.

Earlier report revealed that LPS induced macrophage activation triggers synthesis of nitric oxide (NO), a known pro-inflammatory marker [12]; The most popular drug against filariasis, DEC, being an immunomodulator, has also been reported to enhance pro-inflammatory cytokine expression in vitro in mononuclear cells [13]. Therefore,

Fig. 3 Effect of 4t or DEC (single or in combination) on gene expression of **a** iNOS, **b** TNF- α , **c** TGF- β in PECs of parasitaemic BALB/c mice. Results were analyzed by Kruskal–Wallis test and expressed as median. * $p < 0.001$ compared to group of untreated parasitaemic mice. UD undetected

both of these two agents were used in this study to compare against our proposed drug candidate. Quite in contrast to the pro-inflammatory response of these agents, 4t drug used in this study was found to increase anti-inflammatory and regulatory cytokines gene expression in mice PECs under in vitro system. In addition, expression of pro-inflammatory cytokines and iNOS were found to be down-regulated by administration of 4t drug in in vitro as well as in parasitaemic mice model. Interestingly, 4t drug was also found to suppress the pro-inflammatory effect of DEC in both in vitro and in vivo conditions. Therefore, we propose 4t as a better alternative to DEC, based on two important evidences: since 4t was shown in our earlier study [4] to act directly on the parasite to inflict apoptotic effect which is lethal unlike DEC which is although found to have apoptotic impact but without any direct filaricidal effect in vitro; moreover in the present study, 4t shows beneficial anti-inflammatory effect on host immune system without any possible damage to the host due to augmented pro-inflammatory effect. It can be further advocated for studying the possible conducive effect of this chalcone compound in the chronic cases of lymphatic filariasis, where active inflammatory state in the host results in various long term debilitating complications.

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Compliance with Ethical Standards

Conflict of interest The authors declare no conflict of interest.

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