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## Toll-like receptor-mediated anti-inflammatory action of glaucine and oxoglaucine

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### ABSTRACT

Two isochinoline alkaloids, glaucine and oxoglaucine were investigated for their suggested anti-inflammatory influence concerning nitric oxide and cytokine production. Mouse peritoneal macrophages were stimulated with different Toll-like receptor (TLR) ligands such as LPS for TLR4, zymosan for TLR2 and CpG for TLR9. The alkaloids inhibited TNF- $\alpha$  and IL-6 production induced by these ligands. In regard to IL-12 suppressive effect was registered in the case of CpG stimulation. Glaucine succeeded to enhance LPS and zymosan-induced IL-10 production. The reduction of pro-inflammatory cytokines and increase of anti-inflammatory IL-10 are indicative for their use in different acute and chronic inflammatory diseases.

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### 1. Introduction

Toll-like receptors (TLRs) play a bridging role between innate and adaptive immunity by recognizing conserved microbial structures. TLR binding resulted in dimerization of TLRs [1] leading to activation of IL-1-like pathway dependent upon the adapter MyD88 and further activation of NF- $\kappa$ B [2,3]. TLR2 and TLR4 can signal at the cell surface [4,5], while TLR9 requires acidified environment and its signaling occurs in endosomal compartments [6,7]. TLRs activation might enhance protective inflammation and may also amplify destructive inflammation. Zymosan is a cell wall component of *Saccharomyces cerevisiae* acting through activation of several macrophage receptors including TLR2, dectin-1, the mannose receptor, and complement receptor 3 [8,9], thus triggering various signaling pathways. The simultaneous engagement of different receptors by zymosan synergistically activates inflammatory pathways resulting in an oxidative

burst and an increase of TNF- $\alpha$  production [10]. TLR4 was identified as a lipopolysaccharide (LPS) receptor known to be required for mice to mount effective responses to Gram-negative bacteria in which LPS is a part of the outer cell membrane [11]. Recently, it was shown that immune recognition of bacterial DNA contributes to the host innate inflammatory response. This effect is due to the presence of unmethylated CpG dinucleotides binding to TLR9 since cells from TLR9-deficient mice are unresponsive to CpG stimulation [12].

The macrophages are major participants in innate immunity responses which recognize, phagocytose and eliminate microbial pathogens. Macrophage activation occurs after interaction between pathogen-associated molecular patterns (PAMPs) and TLRs. As a result, it starts a secretion of pro-inflammatory cytokines such as TNF- $\alpha$  important for host defense, whereas anti-inflammatory cytokines, such as IL-10 are produced to limit the inflammation and tissue damage.

There is a permanent need to look for new compounds which can recover the impaired homeostasis. A series of aporphinoid alkaloids from plant origin have been investigated for their cytotoxic properties and potential use as anticancer agents in regard to structure–activity relationships

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[13]. Antiplatelet effect of different aporphins isolated from genus *Magnolia* [14] and from *Rollinia mucosa* [15] has been proved but without exact conclusion, that this effect is structure-related. Glaucine is known for more than 20 years with its spasmolytic, neuropharmacological, hypothermic and blood pressure lowering activities [16–19]. Oxoglaucone expressed antifungal, antiplatelet, vasorelaxing and antileukemic properties [20–22]. Previously, we have investigated the immunological activity of both alkaloids *in vitro* and *in vivo*. We have established their dose-dependent influence on macrophage activity, lymphocyte proliferation and antibody synthesis [23,24]. Oxoglaucone showed beneficial action in adjuvant-induced arthritis [25] and immunorestorative effect in *Candida albicans* infection in mice [26]. The aim of the present study was to elucidate the effect of glaucine and oxoglaucone on macrophage activity in relation to TLRs involvement.

## 2. Experimental

### 2.1. Substances

Glaucine and its structural analogue oxoglaucone (Fig. 1) were obtained via the usual phytochemical procedure from the aerial part of *Glaucium flavum* Crantz (Papaveraceae). A voucher specimen was deposited in the Herbarium of the Institute of Botany, Bulgarian Academy of Sciences (SOM)-BK-86135. Air-dried plant material was extracted exhaustively with 95% ethanol. The crude alkaloid mixture was purified by column chromatography over neutral alumina (Merck, Act. II–III), using a solvent system of increasing polarity and preparative thin layer chromatography. Identification of glaucine and oxoglaucone was carried out by comparison of  $R_f$  values, melting points, mass and infrared spectra data with those of authentic samples. The alkaloids were applied at a concentration of 250 µg/ml, according to our previous work, so to gain full solubility and without cytotoxic effect on murine cells [24].

*Escherichia coli* LPS and zymosan A from *Saccharomyces cerevisiae* were purchased from Sigma (BioChemika, St. Louis, MO, USA), and CpG ODN 1826 from InvivoGen (San Diego, CA, USA).

### 2.2. Isolation of macrophages

In the present experiments C57Bl/6 mice were used. The animals were housed in the Animal facility of the Institute of Microbiology (Sofia), 6–8 weeks of age, 18–20 g body weight, with free access to standard chow and water.

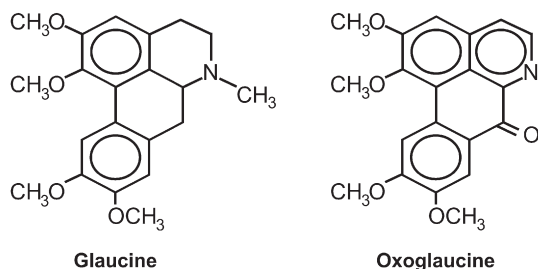


Fig. 1. Chemical structures of glaucine and oxoglaucone.

Peritoneal macrophages were harvested by rinsing the peritoneal cavity with 10 ml of cold medium RPMI-1640 (Biowhittaker®, Cambrex). The cells were washed twice with sterile phosphate-buffered saline (PBS; Cambrex), resuspended at a concentration of  $2 \times 10^6$  cells/ml in RPMI-1640 containing 2 µmol/l L-glutamine (Cambrex) and supplemented with 5% (v/v) fetal calf serum (FCS, Sigma-Aldrich), 10 ml/l penicillin-streptomycin solution (Sigma-Aldrich), 10000 units/ml penicillin G and 10 mg/ml streptomycin. The cells (0.5 ml) were incubated in 24-well culture plates (Greiner, Duesenhofen, Germany) for 1 h at 37 °C in 5% CO<sub>2</sub> and then washed twice to remove the non-adherent cells. The adherent cell population contained more than 90% macrophages positive for F4/80.

### 2.3. Nitric oxide production by macrophages

Peritoneal macrophages were stimulated with LPS (1 µg/ml), zymosan (100 µg/ml) or CpG (20 µg/ml) in the presence or absence of glaucine or oxoglaucone at a concentration of 250 µg/ml for 18 h at 37 °C, 5% CO<sub>2</sub>. The concentration of the stable NO-metabolite, nitrite was determined in the culture supernatants by a standard Greiss reagent (0.1% naphthaethylenediamine dihydrochloride, 1% sulfanilamine in 2.4% H<sub>3</sub>PO<sub>4</sub>) and the absorbance was measured at 540 nm.

### 2.4. Cytokine assays

Peritoneal macrophages were cultivated and stimulated for 24 h as described in the previous paragraph. After centrifugation at 1200 × g for 10 min, the supernatants were collected and frozen at –70 °C. The amounts of TNF-α, IL-6, IL-12 and IL-10 were determined by commercial ELISA kits (Peprotech, London, UK). The detection limit was 50 pg/ml for TNF-α, 60 pg/ml for IL-6, pg/ml for IL-12 and 20 pg/ml for IL-10.

### 2.5. Immunofluorescence analysis

Freshly isolated macrophages were allowed to adhere on slides for 30 min. The cells were incubated for 1 h with oxoglaucone or glaucine at a concentration of 250 µg/ml and after washing were stimulated with LPS (1 µg/ml) for 2 h. To another group of cells, LPS was added simultaneously with the substances for 2 h. After washing the slides were stained with FITC-labeled anti-TNFαR antibody (1:200 diluted Santa-Cruz) for 30 min. The number of cells positive for TNFαR macrophages is examined by fluorescent microscope (magn 1 × 1000) (Boeco, Germany).

### 2.6. Statistics

Statistical significance was evaluated by unpaired *t* test.  $P < 0.05$  was regarded as significant.

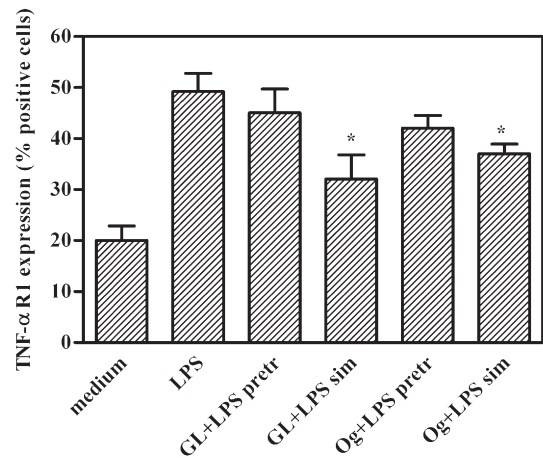
## 3. Results and discussion

In response to an immune challenge, macrophages become activated and produce pro-inflammatory mediators, as their overproduction can result in tissue injury and cellular death. Toll-like receptor activation of macrophages up-regulated the

release of reactive nitrogen intermediates. NO is produced in large quantities during host defense and immunological reactions. Because it has cytostatic properties and is generated by activated macrophages it is likely to have a role in nonspecific immunity. We found that oxoglucine and glucine expressed pronounced inhibitory action on NO release induced by zymosan, LPS and CpG (Supplemental Fig. S1A, B,C). There was no significant difference between both alkaloids in regard to LPS and zymosan stimulation, while glucine had higher suppressive effect on CpG-induced NO release compared to oxoglucine. Macrophage TNF- $\alpha$  production is the principal effect of TLRs signaling. The present experiments showed that comparable levels of TNF- $\alpha$  were observed in macrophage supernatants after LPS and CpG stimulation. This effect decreased 2 fold in the presence of oxoglucine and glucine (Supplemental Fig. S2A,C). To some extent lower inhibitory action expressed the alkaloids upon zymosan stimulation compared to LPS and CpG (Supplemental Fig. S2B).

IL-6 is clearly depicted by its ability to orchestrate transition from innate to acquired immunity. Appropriate control of this immunological switch is essential for the successful resolution of any inflammatory episode, and IL-6 activity appears to be critical for the effective management of acute inflammation [27]. In addition, IL-6 exerts stimulatory effects on T- and B-cells, thus favoring chronic inflammatory responses. IL-6 down regulates LPS-induced TNF- $\alpha$  and IL-1 $\beta$  expression thus it can serve as an endogenous anti-inflammatory mediator. Strategies targeting IL-6 and IL-6 signaling led to effective prevention and treatment of models of chronic inflammatory diseases. We observed that as a result of LPS, zymosan and CpG stimulation peritoneal macrophages released a high amount of IL-6 in the supernatants. Remarkable suppression was caused by the alkaloids as the effect of oxoglucine was greater than that of glucine with respect to LPS and zymosan action (Fig. S2D, E,F). IL-12 is an important type 1 immune activation cytokine. It is known that macrophages and dendritic cells are the major cell types producing this cytokine [28]. The present results concerning macrophage IL-12 production indicated that oxoglucine and glucine failed to change the LPS and zymosan-induced stimulation (Supplemental Fig. S3A,B). In contrast, the effect of CpG was significantly reduced in the presence of both alkaloids (Supplemental Fig. S3C).

IL-10 is an anti-inflammatory cytokine mainly produced by macrophages [29]. During infection it inhibits the activity of Th1 cells, NK cells, and macrophages, all of which are required for optimal pathogen clearance. In consequence, IL-10 can both impair pathogen clearance and attenuate immunopathology. IL-10 release was not influenced by oxoglucine concerning the three Toll ligands used, while glucine additionally enhanced LPS- and zymosan-induced IL-10 production (Supplemental Fig. S3D,E). In contrast, it did not affect CpG-induced IL-10 release (Supplemental Fig. S3F). Macrophage activation is associated with up-regulation of receptor expression. We observed in preliminary experiments that LPS induced the expression of TNF $\alpha$ R1 time-dependently, well expressed 2 h after LPS stimulation. Glucine and oxoglucine did not influence this process if the macrophages were preincubated with the substances. In the case of simultaneous addition of LPS and the alkaloids TNF $\alpha$ R expression was reduced (Fig. 2). These results point that



**Fig. 2.** Expression of TNF- $\alpha$  receptor. Peritoneal macrophages were pretreated (pretr) with glucine or oxoglucine at a concentration of 250  $\mu$ g/ml for 2 h and then stimulated with LPS or simultaneously (sim) incubated with LPS and alkaloids for 2 h. The percentage of positive for TNF- $\alpha$ R was determined by immunofluorescence.

possibly, the substances influenced cytokine production through their specific receptors, at least in regard to TNF- $\alpha$ . In some cases in the course of cytokine production TLRs can cooperate. The simultaneous signaling via TLR4 and TLR2 appeared to induce selective synergy in anti-inflammatory cytokine production by murine dendritic cells [30,31]. Thus, the balance of inflammatory vs anti-inflammatory cytokines proves to be crucial for controlling immune homeostasis. In the light of the present results oxoglucine and glucine appeared to be useful as inhibitors of different inflammatory conditions which deserve further investigations.

#### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fitote.2009.05.016.

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