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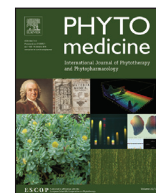

Mitraphylline inhibits lipopolysaccharide-mediated activation of primary human neutrophils

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ABSTRACT

Background: Mitraphylline (MTP) is the major pentacyclic oxindolic alkaloid presented in *Uncaria tomentosa*. It has traditionally been used to treat disorders including arthritis, heart disease, cancer, and other inflammatory diseases. However, the specific role of MTP is still not clear, with more comprehensive studies, our understanding of this ancient herbal medicine will continue growing.
Hypothesis/Purpose: Some studies provided its ability to inhibit proinflamatory cytokines, such as TNF-α, through NF-κB-dependent mechanism. TNF-α primes neutrophils and modulates phagocytic and oxidative burst activities in inflammatory processes. Since, neutrophils represent the most abundant pool of leukocytes in human blood and play a crucial role in inflammation, we aimed to determine the ability of MTP to modulate neutrophil activation and differentially regulate inflammatory-related cytokines.
Methods: To determine the mechanism of action of MTP, we investigated the effects on LPS-activated human primary neutrophils responses including activation surface markers by FACS and the expression of inflammatory cytokines, measured by real time PCR and ELISA.
Results: Treatment with MTP reduced the LPS-dependent activation effects. Activated neutrophils ($CD16^{+}CD62L^{-}$) diminished after MTP administration. Moreover, proinflamatory cytokines (TNF-α, IL-6 or IL-8) expression and secretion were concomitantly reduced, similar to basal control conditions.
Conclusion: Taken together, our results demonstrate that MTP is able to elicit an anti-inflammatory response that modulates neutrophil activation contributing to the attenuation of inflammatory episodes. Further studies are need to characterize the mechanism by which MTP can affect this pathway that could provide a means to develop MTP as new candidate for inflammatory disease therapies.

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Introduction

Uncaria tomentosa (Willdenow ex Roemer & Schultes) DC. (Rubiaceae) is a Peruvian thorny liana, traditionally used in folk medicine to deal with many ailments, such as viral infections, gastric illnesses (gastric ulcers), arthritis and other inflammatory disorders as well as, antitumoral agent (Dietrich et al., 2014; Rojas-Duran et al., 2012; Muller and Kanfer, 2011; Rosenbaum et al., 2010; Garcia-Prado et al., 2007). *U. tomentosa´s* bark contains a series of secondary metabolites, such as oxindole alkaloids and polyphenols (flavonoids, proanthocyanidins, tannins) and small concentrations of other secondary metabolites, such as quinovic acid glycosides, polyhydroxylated triterpenes and saponins (Laus, 2004; Aquino et al., 1990, Aquino et al., 1989).

Most of the alkaloids contained in *U. tomentosa* have been well recognized as phytochemical markers due to their pharmacological activities (Heitzman et al., 2005). Mitraphylline (MTP) is an oxindole alkaloid and the most ubiquitous alkaloid being present in *Uncaria* species (Heitzman et al., 2005). Most of the pharmacological studies have been generally focused on the fractions of either plant species or "crude drug", considered as a preparation from either a single or a mixture of *Uncaria* plants. However, little is known about the bioactivity of isolated compound(s), specifically MTP. We have recently tested the anti-inflammatory and immuno-modulatory action of the MTP on human circulating monocytes (Montserrat-de la Paz et al., 2015); therefore more research is needed to understand completely the

Abbreviations: MTP, Mytraphilline; IL-6, Interleukin 6; IL-8, Interleukin 8; TNF-α, Tumor necrosis factor-α; LPS, lipopolysaccharide.

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role of MTP on leukocytes modulation and its anti-inflammatory properties.

Neutrophils represent the most abundant pool of leukocytes in human blood and play a crucial role in inflammation, stand as the first line of defence of the innate immune system (Mantovani et al., 2011; Brown et al., 2006). Neutrophil-mediated inflammation is the subject of extensive research (Nathan, 2002). Influx of neutrophils into tissues begins by rolling, which is mediated via surface CD62L (L-selectin) interacting with complementary ligands on endothelial cells, and is followed by firm neutrophil adhesion to the endothelium and consequently, transmigration (Lerman and Kim, 2015). Once activated, neutrophils recognize, phagocyte, and kill invading microorganisms, and this function is achieved via release of proteolytic enzymes, pro-inflammatory cytokines as TNF-α, IL-6 or IL-8 and generation of NO (Zimmermann et al., 2015; Jyoti et al., 2014; Wright et al., 2010; Chatterjee et al., 2009). Following killing, it is required an effective resolution of inflammation, and defects of this process is implicated in the pathogenesis of numerous disorders (Roberts et al., 2013; Wright et al., 2010), chronic inflammation (Berry et al., 2010), autoimmunity (García-Romo et al., 2011) and cancer (Kuang et al., 2011).

Therefore, to take advantage of the beneficial effects of MTP from *Uncaria tomentosa*, we evaluated the ability of MTP to modulate LPS-mediated activation on primary human neutrophils. To approach this, we analysed gene and surface markers expression, in addition to the release of pro-inflammatory mediators such as IL-6, IL-8 and TNF-α, in primary human neutrophils, which are strongly involved in the pathogenesis of numerous inflammatory disorders.

Materials and methods

Bark extract

The plant material was collected in the Peruvian forest and was provided by Dr Carlos S. González and was identified in the Botanic Department of the San Lorenzo Chemical Science School from the Asuncion University in Paraguay. The plant extract was obtained and the compound MTP identified as previously described (García-Prado et al., 2007). Briefly, (Supplemental Fig. 1), 500 g of *U. tomentosa* dried inner bark was treated with ammonium hydroxide and extracted with 500 ml of dichloromethane for 3 times. After filtration, the obtained solution was concentrated in vacuo to afford a residue, which was dissolved in a hydrochloric acid solution (3%). Ammonium hydroxide and dichloromethane were added again. After concentration in vacuum, the purified alkaloid fraction was obtained as a brown residue and the yield was 0.1%.

Gas-chromatography/mass-spectrometry (GC/MS) analysis of the alkaloid fraction

Gas-chromatography/mass-spectrometry analysis was performed by using a CARLO ERBA/KRATOS MS 80 RFA apparatus. Helium (99.99%) was the carrier gas (1 ml/min). 1 µl of the sample was dissolved into dichloromethane and injected into the gas chromatograph. The injector and detector temperatures for the gas chromatograph were 275 °C and 325 °C, respectively. The column oven temperature was increased linearly from 230 °C to 300 °C (4 °C/min). The ionization mode was electron impact (EI). NBSL 1 B2 library was used to recognize all derivatives found in the fraction.

Isolation and identification of MTP

The dried residue of alkaloid fraction (0.5 g) was subjected to a silica gel column chromatography, compacted with silica gel with silica gel 0.063–0.2 mm (0.8 × 25 cm), followed by a gradient elution with various mixtures of *n*-hexane, dichloromethane, and methanol (Table 1). All the collected eluents were monitored by thin layer chromatography (TLC). Twenty-five fractions of 15 ml each were collected and 200 mg of white crystal was obtained from the fractions 14–16 and thereafter eluted with a mixture of dichloromethane/methanol (9.5:0.5), which corresponded to 87.2% of the total alkaloids. An isolated compound was obtained by the TLC method (SiO_2, CH_2Cl_2/ MeCO (5:4) Rf: 0.83; ethyl ether/ EtOAc (1:1) Rf: 0.73; CH_2Cl_2/ EtOH (95:5) Rf: 0.68) and visualized as a brown and orange spot with the reagents sulphuric acid/CH3COOH/H2O (1:20:4).

Table 1
Development of the solid–liquid chromatography column corresponding to fraction of the total alkaloids from *U. tomentosa.*

Fractions	Eluents	Eluates
1–4	*n*-Hexane	Mixtures
5–10	Dichloromethane	Mixtures
11–13	*n*-Hexane/Dichloromethane (5:5)	Total alkaloids and mixtures
14–16	Dichloromethane/Methanol (9.5:0.5)	Total alkaloids
17–19	Dichloromethane/Methanol (9:1)	Total alkaloids and mixtures
20–25	Dichloromethane/Methanol (8:2)	Mixtures

EIMS and ^{1}H and ^{13}C NMR experiments were carried out for alkaloid identification by using an AVANCE 500 spectrophotometer. The following 2D NMR experiments COSY-DQF, (^{1}H–^{13}C)–HSQC, (^{1}H–^{13}C)–HMBC, and NOESY correlation was used to elucidate its structure. Furthermore, the ^{15}N chemical shifts of the isomeric oxindole alkaloids, (^{1}H–^{15}N)–HMBC, was necessary to facilitate its characterization. The solvent used for 70 NMR spectra was CDCl3 (García-Gimenez et al., 2010).

Blood collection and neutrophil isolation

This study was conducted according to the guidelines of good clinical practice. Peripheral venous blood was isolated from healthy adult volunteers (< 35 years old) at the University Hospital Virgen del Rocio at Seville. The investigation conformed to the principles outlined in the Helsinki Declaration of the World Medical Association. Neutrophils were isolated by dextran sedimentation in a Ficoll Histopaque gradient (Sigma-Aldrich Chem., St. Louis, MO, USA) and erythrocytes were removed by hypotonic lysis. The purity of neutrophils preparation was > 97% by trypan blue exclusion. Following isolation, the cells were suspended in a RPMI 1640 medium supplemented with L-glutamine, penicillin, streptomycin and 1% heat-inactivated fetal bovine serum. Neutrophils were seeded at a density of 3×10^{6} cells/ml. Cells were treated with 0.1 µg/ml LPS from *E. Coli 055:B5* (Sigma-Aldrich®, St Louis, MO, USA) in presence or absence of the MTP (25 µM) for 6 h.

Cytotoxicity assay

Neutrophils seeded in 96-well plates (1×10^{5} cells/well) were incubated in presence or absence of different MTP concentrations for 6 and 24 h. At the end of the exposure time, the effect on cell growth/viability was analyzed by MTT colorimetric assay (Montserrat-de la Paz et al., 2012). Cell survival was measured as the percentage of absorbance compared with that obtained in control cells (non-treated cells).

Nitrite and cytokine production

Cells in 24-well plates were treated (or untreated) with MTP (25 µM), and 30 min later stimulated with LPS (0,1 µg/ml) for 6 h.

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