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Manumycin A downregulates release of proinflammatory cytokines from TNF alpha stimulated human monocytes

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ABSTRACT

Macrolide antibiotics such as azithromycin or clarithromycin are known to have potent anti-inflammatory and immunomodulatory effects but these properties cannot be widely used due to a risk of bacterial resistance. We studied another polyketide antibiotic, structurally related manumycin A known as a streptomycete derived farnesyltransferase inhibitor with limited antibacterial effects, with respect to its potential regulation of mRNA expression of several genes associated with proinflammatory responses. Downregulation of mRNA for IL-6, TLR-8, IL-1 beta and IL-10 was found in THP-1 cells after 4 h stimulation with TNF alpha in the presence of manumycin A and downregulated TLR-8 and EGR-1 genes were observed after 8 h. Among the genes upregulated in response to manumycin were HMOX-1, TNFRSF10A, IL-1R1, TICAM2, NLRP12 after 4 h and only IL-1R1 after 8 h.

Furthermore, manumycin A was found to inhibit IL-1beta, IL-6, and IL-8 production in TNF alpha stimulated THP-1 cells and peripheral blood monocytes in a dose dependent manner (0.25–1 μM of manumycin A) without affecting cell viability. Cell viability of blood monocytes decreased by about 30% at manumycin A doses of 2–5 μM. Manumycin A also inhibited IL-18 release from THP-1 cells, while in cultures of blood monocytes, this cytokine was not detectable.

That manumycin A mediated downregulation of proinflammatory genes in human monocytes confirmed by a measurement of cytokine levels in culture supernatants, together with a very limited effect on cell viability, might suggest potential anti-inflammatory properties of this polyketide antibiotic.

1. Introduction

Polyketide antibiotics, including macrolides such as azithromycin or clarithromycin, are known to exert potent anti-inflammatory and immunomodulatory effects beyond their purely antibacterial action [1–3]. The mechanisms of their biological activities are still being investigated [4] but inhibition of neutrophil and lymphocyte migration [5] and suppression of proinflammatory cytokines, such as IL-1, IL-6, and TNF alpha might be preferentially involved [6]. For the most part, azithromycin has been found to be effective for the treatment of neutrophilic airways disease [7] and inhibition of IL-5 release from Th2 cells of asthmatic children [8]. Furthermore, an experimental model of a bleomycin induced pulmonary fibrosis used to resemble idiopathic pulmonary fibrosis indicated the beneficial effect of azithromycin in the fibrosing process [9]. On a molecular level, macrolides seem to affect the signaling pathways of transcription factors which regulate a number of pro-inflammatory and/or pro-fibrotic genes [10]. Although the anti-inflammatory effects of macrolides are well documented, obviously the high risk of bacterial resistance is the main obstacle to use them for targeting immune cells [11]. In this respect, cognate substances with less potent antibacterial properties which are not generally used as antibacterials might represent a very attractive tool to modulate immune responses. One of the potential candidates is manumycin A, a streptomycete derived farnesyltransferase inhibitor with a capacity to inhibit Ras and downregulate the expression of adhesion molecules required for cell-to-cell interactions of T lymphocytes with mesenchymal stromal cells [12]. In human endometrial stromal cells, manumycin A partially inhibited phospholipase D1 expression and decidua formation in response to cAMP treatment [13]. In an experimental model of a murine heterotopic heart transplant model, manumycin reduced inflammatory cell recruitment into the allograft [14]. Manumycin A also prevented the development

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of atherosclerosis in apolipoprotein E (apoE)-deficient mice fed a high-fat diet by reducing superoxide production and nitrotyrosine immunoreactivity in the aorta [15]. The aim of our study was to characterize in stimulated human monocytes the effect of manumycin A on gene expression of multiple molecules involved in the regulation of immune responses and evaluate the release of proinflammatory cytokines.

2. Materials and methods

2.1. Isolation of PBMCs and stimulation of monocytes

PBMCs obtained from healthy volunteers were purified using standard Ficoll-Paque gradient centrifugation. Briefly, 3 ml of Ficoll-Paque gradient was pipetted into a 15-ml centrifuge tube. The EDTA blood was diluted 1:1 in phosphate-buffered saline (PBS) and carefully layered over the Ficoll-Paque gradient (3 ml/tube). The tubes were centrifuged for 30 min at 580 × *g*. The cell interface layer was harvested carefully and the cells were washed once in PBS (for 10 min at 180 × *g*) and were washed once again in RPMI-1640 medium (Sigma–Aldrich, St. Louis, MO, USA), (for 10 min at 180 × *g*). Monocytes from PBMCs were separated by pluriBead Cell separation technology (PluriSelect, Germany). This technology uses non-magnetic microparticles (beads) coated with monoclonal antibodies that recognize specific molecules on the target cell surface (CD14 in this case). Monocytes bind to anti-CD14 on the surface of microparticles and other cells are washed away. At the end of the washing procedure, the monocytes are detached from the microparticles with a detachment buffer. We measured the purity of monocytes after separation by flow cytometry.

Monocytes were removed into 96 or 6-well tissue culture plates (Costar, Corning, NY, USA) at a density of 2×10^6 cells/ml and stimulated with TNF alpha (20 ng/ml) in the presence or absence (positive control) of manumycin A (Sigma–Aldrich, St. Louis, MO, USA). Unstimulated cells were used as controls. Trypsin-EDTA solution (Sigma–Aldrich, St. Louis, MO, USA) was used for detachment of adherent cells from the surface of culture plate before measuring monocytes.

2.2. Cell culture conditions

Human monocytic leukemia cells THP-1 (purchased from American Type Culture Collection, ATCC) were cultured in RPMI-1640 medium (Sigma–Aldrich, St. Louis, MO, USA), supplemented with 10% heat inactivated fetal calf serum (FCS), L-glutamin, penicillin and streptomycin (Sigma–Aldrich). Cells were removed into 6-well tissue culture plates (Costar, Corning, NY, USA) at a density of 2×10^6 cells per 2 ml per well and cultured under a 5% CO_2 atmosphere at 37 °C. Cells were stimulated with TNF alpha (20 ng/ml) under serum free conditions in the presence and absence of manumycin A (Sigma–Aldrich, St. Louis, MO, USA). Unstimulated cells and those preincubated with synthetic caspase-1 inhibitor Ac-YVAD-CHO (Calbiochem, La Jolla, CA, USA) were used as controls.

2.3. Viability

The standard method of Trypan Blue Dye Exclusion was used for measuring cell viability of blood monocytes and THP-1 cells. Trypan blue (0,4%) was mixed with an equal volume of cells containing solution. Viable cells, due to their intact membranes, exclude the trypan blue stain and non-viable cell with permeable membrane are stained dark blue. The results of staining were detected on a Vi-cell analyzer (Beckman Coulter).

2.4. RNA extraction

After the cell harvesting, cells were immediately centrifuged 5 min at 2000 rpm. Supernatants were separated and frozen at −20 °C. The total RNA was extracted with an RNeasy Plus Mini Kit (Qiagen, Germany) according to manufacturer's instructions. The RNA quality was measured with spectrophotometer at absorbancies 230, 260 and 280 nm. Samples with a ratio A230/260 > 1.7 and A260/280 > 1.9 were considered suitable for quantitative real-time PCR analysis (qRT-PCR).

2.5. Quantitative RT-PCR (qRT-PCR)

The qRT-PCR analysis was performed using RT2 Profiler PCR Arrays (Common Cytokines Array Kit, Cat. No. PAHS-021Z) from SABiosciences (Qiagen, USA) on an ABI 7900HT instrument. This kit contains 84 different specific primers for immunoregulation related genes, plus controls for genomic contamination and reverse transcription and positive PCR controls (Table 1). Data were evaluated with a web-based program provided by the manufacturer.

2.6. Immunoluminiscent detection of cytokines by Luminex

IL-1beta, IL-6, and IL-8 were measured in cells culture supernatants by a Fluorokine MAP human base kit A (R&D Systems) using the Luminex 100 System (Luminex Corporation, Austin, Texas, USA). In the first step, 50 μl of the samples/standards were incubated with 50 μl of microparticles for 3 h at RT on a horizontal orbital microplate shaker. After washing the unbound substances, we added 50 μl of the secondary antibodies conjugated with biotin to each well and we incubated the samples/standards for another 1 h. After the incubation, we washed out the unbound secondary antibodies and added 50 μl of the Streptavidin-PE. After 30 min of incubation, we washed the samples/standards and resuspended the microparticles in 100 μl of wash buffer. Samples/standards were read using a Luminex analyzer.

2.7. IL-18 ELISA

IL-18 levels were measured in supernatants of THP-1 cells or human monocytes stimulated with TNF alpha in the presence of manumycin A using a sandwich ELISA (MBL, Nagoya, Japan). Briefly, the samples and standards were incubated in 96 well plates precoated with anti-human IL-18 monoclonal antibody for 60 min. After washing, a peroxidase conjugated anti-human 18 monoclonal antibody recognizing a different epitope of IL-18 was added. After 60 min of incubation and another washing, the peroxidase substrate TMB/H_2O_2 was added. The reaction was stopped after 30 min by an acid solution (2N H_2SO_4) and the optical density was measured at 450 nm using a microplate reader. The concentration of human IL-18 was calibrated from a dose response curve based on reference standards.

2.8. Statistics

Student's *t*-test and ANOVA statistics were used for the comparison of protein data and functional assays. A *p*-value less than 0.05 was considered significant. Data from qRT-PCR were evaluated with a web-based program of the company (http://www.sabiosciences.com/pcr/arrayanalysis.php). Briefly, the evaluation of relative gene expression was based on ΔΔCT algorithm and Student's *t*-statistics. Gene expression was normalized to a mean expression of housekeeping genes B2 M (beta-2-microglobulin), HPRT1 (hypoxanthine phosphoribosyltransferase 1), RPL13A (ribosomal protein L13a), GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and ACTB (actin beta). Relative gene quantification was evaluated by