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Intensity of macrolide anti-inflammatory activity in J774A.1 cells positively correlates with cellular accumulation and phospholipidosis

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ABSTRACT

Some macrolide antibiotics were reported to inhibit interleukin-6 (IL6) and prostaglandin-E2 (PGE_2) production by bacterial lipopolysaccharide (LPS) stimulated J774A.1 cells. Macrolides are also known to accumulate in cells and some were proven inducers of phospholipidosis. In the present study, with a set of 18 mainly 14- and 15-membered macrolides, we have investigated whether these macrolide induced phenomena in J774A.1 cells are connected.

In LPS-stimulated J774A.1 cells, the extent of inhibition of proinflammatory markers (IL6 and PGE_2) by macrolides significantly correlated with their extent of accumulation in cells, as well as with the induction of phospholipidosis, and cytotoxic effects in prolonged culture (with correlation coefficients (R) ranging from 0.78 to 0.93). The effects observed were related to macrolide binding to phospholipids (CHI IAM), number of positively charged centres, and were inversely proportional to the number of hydrogen bond donors. Similar interdependence of effects was obtained with chloroquine and amiodarone, whereas for dexamethasone and indomethacin these effects were not linked.

The observed macrolide induced phenomena in J774A.1 cells were reversible and elimination of the macrolides from the culture media prevented phospholipidosis and the development of cytotoxicity in long-term cultures.

Based on comparison with known clinical data, we conclude that LPS-stimulated J774A.1 cells in presented experimental setup are not a representative cellular model for the evaluation of macrolide anti-inflammatory potential in clinical trials.

Nevertheless, our study shows that, at least in *in vitro* models, binding to biological membranes may be the crucial factor of macrolide mechanism of action.

1. Introduction

Macrolide antibiotics with a 14- and 15-membered lactone ring are widely used in the clinic. Isolated in 1952 from the actinomycete *Saccharopolyspora erythraea*, erythromycin A was the first member of the class and was succeeded by several semi-synthetic macrolides of which azithromycin has been the most successful commercially [1].

In addition to their therapeutic efficacy as antimicrobials, standard macrolide antibiotics have been reported to exert significant anti-inflammatory/immunomodulatory activity [2,3]. So far, the most pronounced clinical effects have been observed in chronic pulmonary inflammatory diseases with dominant neutrophilic infiltration, in which macrolides inhibit both accumulation of neutrophils and proinflammatory cytokine release in inflamed tissues. Nowadays, macrolides are used as a standard treatment of diffuse panbronchiolitis and cystic fibrosis, and their potential uses in chronic obstructive pulmonary disease, chronic sinusitis, asthma,

Abbreviations: AGP, alpha-acid glycoprotein; AK, adenylate kinase; AMP, artificial membrane permeability; CHI, chromatographic hydrophobicity index; cpd, compound; DMSO, dimethyl-sulphoxide; HSA, human serum albumin; IAM, immobilized artificial membrane; IL6, interleukin-6; LPS, lipopolysaccharide; NBD-PE, nitrobenzoxadiazolyl labelled phosphatidylethanolamine; ns, nonstimulated cells; PCA, principal component analysis; PGE_2, prostaglandin E2; PK, pharmacokinetics; SD, standard deviation; TNFα, tumour necrosis factor-alpha.

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bronchiectasis, and bronchiolitis obliterans have also been reported [4].

Many *in vitro* models for the evaluation of macrolide anti-inflammatory activity have been described [2,5]. J774A.1 is a widely used murine monocyte–macrophage cell line in which standard antimicrobial macrolides were found to inhibit interleukin-6 (IL6) and prostaglandin E2 (PGE_2) production upon stimulation with bacterial lipopolysaccharide (LPS) [6]. In the same cell line azithromycin affects membrane traffic and endocytosis [7] and like other standard macrolide antibiotics highly binds to lipids [8]. In addition, in cultured fibroblasts azithromycin was reported to induce phospholipidosis [9]. This phenomenon was proven reversible *in vivo* when the circulating azithromycin concentration dropped down following treatment cessation [10,11].

Macrolides are also known to accumulate in tissues and cells. During 3 h incubation with cells some macrolides can reach intracellular concentrations up to 500 fold higher than extracellular [12]. In our recent study, using 48 rationally designed macrolides, we have analyzed the effect of macrolide structure and molecular properties on their cellular accumulation and retention [13]. We found that in cellular pharmacokinetics macrolide structure behaves as a complex unit that cannot be treated as a sum of smaller structural fragments. Thus, macrolide cellular accumulation is mostly driven by whole molecular properties reflecting molecular charge, binding to phospholipids and lipophilicity.

In the present study we have compared 18 macrolides, mainly with 14- and 15-membered ring, to analyze the potential connection between macrolide accumulation, their interaction with lipids, and their anti-inflammatory actions in a widely used cellular model of LPS-stimulated J774A.1 cells. In another study we showed that the site of azithromycin action in J774A.1 cells is most likely at the level of cytoplasmic phospholipase A2 (cPLA2) (Banjanac M. et al., unpublished results). The present study aimed to evaluate LPS-stimulated J774A.1 cells as a model for screening macrolide anti-inflammatory activity, as well as to investigate the mechanisms leading to the reported activities.

2. Materials and methods

2.1. Substances

Clarithromycin was purchased from US Pharmacopeia, roxithromycin, erythromycin, indomethacin, dexamethasone, chloroquine and amiodarone from Sigma, tylosin from Wako Chemicals and azithromycin from PLIVA. All other macrolides were synthesized internally in GlaxoSmithKline Research Centre Zagreb. All compounds were dissolved in dimethyl-sulphoxide (DMSO, Sigma), except chloroquine which was dissolved in deionized water (MilliQ, Millipore). Macrolides were dissolved at a concentration of 50 mM, indomethacin and dexamethasone at 10 mM, amiodarone at 25 mM and chloroquine at 12.5 mM.

2.2. Cells

Murine macrophage cell line J774A.1 (ATCC, TIB-67) was grown in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Invitrogen) with 1% Glutamax (Gibco, Invitrogen), 10% fetal bovine serum (FBS, Biowest). Cells were passaged twice weekly and used in passages 8–18.

2.3. Anti-inflammatory activity in J774A.1 cells

Two days before the experiment cells were seeded in 24-well plates at a density of 10^5/mL/well. On the day of the experiment, cells were given fresh medium containing the test substances at concentrations of 50, 25 and 12.5 μM, unless stated otherwise, and incubated at 37 °C, 5% CO_2 for 2 h. LPS (*E. coli*, 0111 B4, Sigma) was added at a final concentration of 1 μg/mL, and incubation continued for the next 6 h. Supernatants were collected and frozen until analysis.

PGE_2 concentrations were determined by competitive ELISA kit (Correlate EIA, Assay Designs). Tumour necrosis factor-alpha (TNFα) and IL6 were determined by sandwich ELISA using capture and detection antibodies purchased from R&D Systems, and performed according to the manufacturer's instructions. Optical density was measured at 450 nm using a microplate reader (SpectraMax 190, Molecular Devices). Concentrations of PGE_2 and cytokines were determined by interpolation from standard curves with SoftMax Pro v4.3.1 software (Molecular Devices). Since some of the treatments showed stimulatory rather than inhibitory effect, in order to analyze correlations with other parameters, results were expressed as fold change over positive control (LPS-stimulated cells treated with vehicle – 0.1% solvent DMSO). Values for IL6 and PGE_2 measured in negative controls (unstimulated cells treated with 0.1% DMSO) were 0.03 ± 0.04 and 0.02 ± 0.01 fold over positive control, respectively. Compounds were considered active if they showed high, moderate or low activity by achieving ≤0.25, 0.26–0.50, 0.51–0.75 fold over positive control, respectively. In other experiments, for the ease of understanding, results of IL6 and PGE_2 were expressed as % inhibition.

In some experiments p40 subunit of interleukin 12 (IL12p40), granulocyte–macrophage colony stimulating factor (GM-CSF) and chemokines CXCL1, CXCL2, CCL2 and CCL5 have been measured by ELISA in the similar manner as described for IL6 and TNFα.

2.4. Cellular accumulation

Cellular accumulation and retention experiments were performed as described previously [13]. Briefly, cells seeded in 12-well plates, were incubated for 3 h with 10 μM compounds in DMEM medium and 1 μg/mL LPS. To determine cellular accumulation, cells were washed and lysed in 0.5% TritonX-100 (Sigma) in deionized water. Concentrations in samples were determined by the liquid chromatography with tandem mass spectrometry (LC–MS/MS) method, as described previously [14]. Intracellular concentration was normalized to total protein content determined by the bicinchoninic acid assay (BCA, Pierce).

Accumulation was expressed relative to the standard macrolide, azithromycin (100%). Compounds were considered to have accumulated to low, moderate or high extent if they achieved 30–49, 50–99 or >100%, respectively, of the accumulation of azithromycin.

2.5. Phospholipidosis assay

Induction of phospholipidosis was measured as an increase in incorporation of phosphatidylethanolamine labelled with fluorescent nitrobenzoxadiazolyl (NBD-PE, Molecular Probes). Cells were seeded in black-walled 96-well plates, and incubated with test substances at concentrations of 25 or 50 μM together with filtered 2 μM NBD-PE with or without LPS (1 μg/mL) for 17 h at 37 °C, 5% CO_2. Cells were carefully washed twice with phosphate buffered saline (PBS, Sigma) and lysed by freezing in 0.5% TritonX-100 in deionized water. Samples were thawed and fluorescence (Ex: 480 nm, Em: 530 nm) was measured on a Victor^2 Wallac plate reader. Results were expressed as fold change over vehicle-treated control (solvent DMSO).

Compounds were considered to be weak, moderate or strong inducers of phospholipidosis if they achieved 1.20–1.49, 1.50–1.99, >2.00 fold change, respectively, over control.