In vivo efficacy of telithromycin on cytokine and nitric oxide formation in lipopolysaccharide-induced acute systemic inflammation in mice

Kristina Lotter¹*, Klaus Höcherl¹, Michael Bucher² and Frieder Kees¹

¹Department of Pharmacology und Toxicology, University of Regensburg, Universitätsstr. 31, 93040 Regensburg, Germany; ²Department of Anesthesiology, University of Regensburg, Franz-Josef-Strauß-Allee 11, 93053 Regensburg, Germany

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Objectives: The ketolide telithromycin represents a new subclass of 14-membered semisynthetic macrolides. Because there is evidence that traditional macrolides such as roxithromycin exert anti-inflammatory activity, we investigated the anti-inflammatory action of telithromycin against lipopolysaccharide (LPS)-induced acute systemic inflammation in mice in comparison with roxithromycin.

Methods: CD-1 mice were injected intraperitoneally with LPS (1 mg/kg), and the effects of pretreatment with a single intraperitoneal dose of telithromycin (150 mg/kg) or roxithromycin (50 mg/kg) for 2 h on the expression and formation of tumour necrosis factor alpha (TNFα), interleukin-1 beta (IL-1β), interferon gamma (IFNγ) and inducible nitric oxide synthase (NOS-II) as well as nitric oxide (NO) were analysed at different time points after LPS-treatment. Cytokine and NOS-II mRNA abundance was examined using real-time RT–PCR. Tissue cytokine levels were determined by enzyme-linked immunosorbent assay kits (ELISA); NO levels were measured by colorimetric assay kits.

Results: Pretreatment of mice with telithromycin as well as roxithromycin similarly attenuated the LPS-induced expression and formation of TNFα, IL-1β and IFNγ. Furthermore, the LPS-induced increase of NOS-II mRNA and the formation of NO were clearly diminished.

Conclusion: These results suggest that the ketolide telithromycin has anti-inflammatory properties like conventional macrolides due to inhibition of the production of proinflammatory cytokines, which leads to a decreased formation of NO in LPS-treated mice. Our data indicate that ketolides may have beneficial therapeutic effects independent of their antibacterial activity.

Keywords: telithromycin, lipopolysaccharide, tumour necrosis factor alpha, interleukin-1 beta, interferon gamma, nitric oxide

Introduction

Macrolides, such as roxithromycin, are a well-established class of antibacterial agents which are active against many Gram-positive and some Gram-negative bacteria. Besides their antibacterial activity, these compounds are reported to exert anti-inflammatory and immunomodulatory activity in vitro and in vivo. It has been described previously that macrolides affect several steps of the inflammatory process, such as migration of neutrophils, modulation of oxidative burst and production of cytokines. In particular, it has been shown that macrolides reduce the production of proinflammatory cytokines, like tumour necrosis factor alpha (TNFα) and interleukin-1β (IL-1β), in response to lipopolysaccharide (LPS) in vitro as well as in vivo using a low-dose, long-term scheme of macrolide treatment. In addition, macrolides have beneficial clinical effects in the treatment of some inflammatory airway diseases in humans, including diffuse panbronchiolitis and chronic sinusitis. In this regard, it has been suggested that the anti-inflammatory action, but not the antimicrobial activity of macrolides, is responsible for the clinical effectiveness of these compounds in chronic inflammatory disorders.

Telithromycin belongs to the group of ketolide antibiotics, which are derived from 14-membered macrolides by replacement...
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of the sugar cladinose at position C3 with a keto group. This alteration resulted in both improved pharmacokinetic properties and an improved spectrum of activity against respiratory tract pathogens compared with erythromycin. In particular, telithromycin was shown to penetrate into human bone and to exhibit excellent activity against macrolide-resistant streptococci. Recently, it has been reported that the ketelololithromycin inhibits LPS-stimulated TNFα and IL-1α formation as well as Shiga toxin-stimulated cytokine production in human monocytes. However, it is unclear whether telithromycin has similar effects on cytokine formation in vivo as well.

It is generally accepted that the first mediators in the cascade of LPS-induced events are cytokines, like TNFα, IL-1β and in some cases interferon gamma (IFNγ). The release of cytokines leads to a subsequent induction of enzymes and signalling proteins in affected tissues and cells. Among these pro-inflammatory enzymes, the inducible form of nitric oxide synthase (NOS-II), which is responsible for increasing levels of nitric oxide (NO), is known to be involved in the pathogenesis of inflammatory processes. In the respiratory tract, for example, an overproduction of NO and its metabolites, such as peroxynitrite, may cause deleterious effects, like injury to endothelial cells, leading to vascular leakage in lung.

Recently, macrolides have been shown to suppress NO generation both in vitro and in vivo in response to inflammatory stimuli, which may account for the clinical effectiveness of macrolides in inflammatory airway diseases.

Because only limited information is available about ketolides with regard to their anti-inflammatory properties, we investigated the effect of a single-dose treatment of mice with telithromycin in comparison with roxithromycin on the formation of proinflammatory cytokines and NO using an in vivo model of acute LPS-induced systemic inflammation.

Materials and methods

Animal model and treatment

All animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and German laws relating to the protection of animals and were approved by the local ethics committee. Male CD-1 mice (Charles River, Sulzfeld, Germany) weighing 30–40 g were used for this study. Animals were housed in cages with food and water ad libitum. The light cycle was controlled automatically (on at 7:00 a.m. and off at 7:00 p.m.), and the room temperature was thermostatically regulated to 22 ± 1°C. Prior to the experiments, animals were housed under these conditions for 3–4 days to become acclimatized. Mice were divided into groups consisting of five mice each. Animals were slightly anaesthetized with sevoflurane, and 1 mg/kg LPS (from Escherichia coli O111:B4, Sigma, Deisenhofen, Germany) dissolved in saline was injected intraperitoneally (0.5 mL per mouse). A suspension of 50 mg/kg roxithromycin (Sigma, Deisenhofen, Germany) or 150 mg/kg telithromycin (a generous gift from Aventis Pharma, Bad Soden, Germany) in saline was administered intraperitoneally 2 h before LPS injection (0.25 mL per mouse). Control groups received an equal volume of saline instead of LPS and antibiotics, respectively. Mice were sacrificed by decapitation during anaesthesia with sevoflurane 1, 2 or 6 h after LPS injection. Blood was collected in EDTA tubes and centrifuged at 4000 rpm for 10 min at 4°C. Plasma was obtained and stored at –20°C until used. Heart, lungs, kidneys and liver were quickly removed, frozen in liquid nitrogen and stored at –80°C until use.

RNA extraction and reverse transcription

Total RNA from heart, lung, kidney and liver was isolated using TRIzol Reagent (Invitrogen, Karlsruhe, Germany) following the manufacturer’s instructions and was quantified spectrophotometrically.

Reverse transcription was performed according to standard protocols in a total volume of 22 μL containing 2 μg of total RNA, 0.5 mg/mL oligo(dT)15 primer (Promega, Madison, USA), 20 U of RNAsin (Promega, Madison, USA), 2.5 mM dNTP (Promega, Madison, USA), 200 U of Moloney murine leukaemia virus Reverse Transcriptase (M-MLV RT) (Invitrogen, Karlsruhe, Germany) and manufacturer’s 5x RT Buffer. cDNA was stored at –20°C until use.

Real-time PCR analysis

Expression of TNFα, IL-1β, IFNγ, NOS-II and β-actin mRNA was evaluated by real-time PCR using the LightCycler System (Roche Diagnostics, Mannheim, Germany). All PCRs were performed in a total volume of 20 μL using the LightCycler FastStart DNA Master SYBR Green I Kit (Roche Diagnostics, Mannheim, Germany). Each reaction contained 2 μL of cDNA, 3.0 mM (TNFα, IL-1β, β-actin) or 5.0 mM (IFNγ, NOS-II) MgCl2, 1 μL each of sense and antisense primer (10 pmol/μL) and 2 μL of Fast Starter Mix (containing buffer, dNTPs, SYBR Green and hotstart Taq polymerase). After an initial denaturation step at 95°C for 10 min, temperature cycling with a total of 40 cycles was initiated. Each cycle consisted of a denaturation phase at 95°C for 10 s, an annealing phase at 58°C for 30 s and an elongation phase at 72°C for 30 s. Amplification was followed by melting curve analysis to verify the correctness of the amplicon. A negative control with water instead of cDNA was run within every PCR to assess specificity of the reaction. To verify the accuracy of the amplification, PCR products were further analysed on ethidium bromide-stained 2% agarose gel. For data analysis, LightCycler software version 3.5 was used. Results are given as a ratio of the amount of TNFα, IL-1β, IFNγ and NOS-II mRNA to that of β-actin mRNA. The following primers were used: TNFα (NM_013693) sense: CCC GGG CTC AGC CTC TTC TCA TTC, antisense: GGA TCC GGT GGT TTG CTA CGA CGT (201 bp); IL-1β (NM_008361) sense: TCT CGC AGC AGC ACA TCA, antisense: CAC ACA CCA GCA GGT TAT (197 bp); IFNγ (NM_008337) sense: CAC AGT CAT TGA AAG CCT, antisense: AGA CTG CAA AGA CTC TGA (169 bp); NOS-II (NM_010927) sense: GCT TAG AGA ACT CCA C, antisense: GCC GCC CTC GTA GGT GAG C (453 bp) and β-actin (NM_007393) sense: CCG CCC TAG GCA CCA GGG TG, antisense: GCC TGG GTT GGT GTA GGT CCA TCA AAA (285 bp).

Protein extraction and determination

Tissues were homogenized in phosphate buffered saline (Sigma, Deisenhofen, Germany) using an ultraturrax for 60 s in an ice-cold water bath. The homogenates were then centrifuged for 10 min at 10 000 rpm at 4°C to remove debris. The supernatants were collected and protein levels were measured by the bicinchoninic acid (BCA) assay kit (Sigma, Deisenhofen, Germany) using bovine serum albumin (BSA) as standard.

Assay for cytokines and NO (NO2/NO3)

TNFα, IL-1β and IFNγ levels in tissues were assayed by using enzyme-linked immunosorbent assay kits (R&D Systems, Germany) and manufacturer’s 5x RT Buffer. cDNA was stored at –20°C until use.
Telithromycin and LPS-induced systemic inflammation

Injection of LPS (1 mg/kg) increased TNFα mRNA expression in heart, lung, kidney and liver after 1, 2 and 6 h (Figure 1a). Maximal stimulation was observed 1 h after LPS injection in all organs. LPS injection increased IL-1β mRNA abundance in heart, lung, kidney and liver after 1, 2 and 6 h (Figure 1b). Maximal stimulation was observed after 1 h in heart and kidney and after 2 h in lung and liver. LPS injection increased IFNγ mRNA expression in heart, lung, kidney and liver after 2 and 6 h (Figure 1c). Maximal stimulation was observed after 6 h. Induction of NOS-II mRNA by LPS injection was increased after 2 h only in liver and kidney and after 6 h in all organs (Figure 1d).

We further investigated the tissue concentration of cytokines and NO in lung and liver. Injection of LPS (1 mg/kg) increased tissue concentration of TNFα and IL-1β after 2 h in lung and liver (Figure 2a and b). IFNγ and NO were increased following LPS injection after 6 h in lung and liver (Figure 2c and d).

Influence of roxithromycin and telithromycin on LPS-induced mRNA expression and on tissue concentration of TNFα, IL-1β and IFNγ

Roxithromycin (50 mg/kg) and telithromycin (150 mg/kg) were examined for their effects on the production of cytokines after administration of LPS (1 mg/kg). LPS-induced TNFα mRNA abundance, measured 1 h after treatment with LPS, was attenuated by pretreatment with roxithromycin or telithromycin in all organs examined (Figure 3a). In contrast, LPS-induced IL-1β mRNA abundance, measured 2 h after administration of LPS, was attenuated by pretreatment with roxithromycin or telithromycin only in lung, kidney and liver, but not in the heart (Figure 3b). LPS-induced IFNγ mRNA abundance, measured 6 h after administration of LPS, was attenuated by pretreatment with roxithromycin or telithromycin in all organs examined (Figure 3c). We further investigated tissue concentrations of cytokines in lung and liver. TNFα and IL-1β levels were determined 2 h and IFNγ levels 6 h after LPS injection. Pretreatment with roxithromycin or telithromycin clearly attenuated the LPS-induced formation of TNFα in lung and liver (Figure 4a).

Figure 1. Time course of LPS-induced TNFα (a), IL-1β (b), IFNγ (c) and NOS-II (d) mRNA expression in heart, lung, kidney and liver. Real-time PCR data are normalized to β-actin mRNA. Data are mean ± SEM of 5 animals in each group; stars indicate P < 0.05 compared to 0 h.
In contrast, pretreatment with roxithromycin or telithromycin attenuated the LPS-induced formation of IL-1β only in lung, but not in liver (Figure 4b). LPS-induced formation of IFNγ was clearly attenuated by pretreatment with roxithromycin or telithromycin in lung and liver (Figure 4a).

Influence of roxithromycin and telithromycin on LPS-induced NOS-II mRNA expression and NO formation

Roxithromycin (50 mg/kg) and telithromycin (150 mg/kg) were further examined for their effects on the generation of NO after treatment with LPS (1 mg/kg). Pretreatment with roxithromycin or telithromycin clearly attenuated LPS-induced NOS-II mRNA expression, measured 6 h after LPS injection, only in heart, lung and kidney but not in liver (Figure 5a). In addition, pretreatment with roxithromycin or telithromycin attenuated the LPS-induced formation of NO, measured 6 h after LPS injection, only in lung but not in liver (Figure 5b). Therefore, we investigated NO generation also in the plasma. Pretreatment with roxithromycin or telithromycin strongly inhibited the LPS-induced formation of NO in plasma, measured 6 h after LPS injection (Figure 5b).

Discussion

In the present study, the anti-inflammatory activity of a single-dose administration of telithromycin in comparison to roxithromycin with regard to their ability to reduce the production of proinflammatory mediators has been investigated in vivo. Intraperitoneal injection of LPS was used as a model for an acute systemic inflammation. Our present in vivo data strongly suggest that attenuation of the induction of the cytokines TNFα, IL-1β and IFNγ and the subsequent induction of NO formation in response to LPS may, in part, account for clinical efficacy of ketolides in the treatment of inflammatory diseases just as well as conventional macrolides.

In accordance to our previous observations, we found that LPS caused a time-dependent increase of TNFα, IL-1β and IFNγ mRNA in all tissues examined.28–30 Compared with TNFα and IL-1β mRNA, the increase of IFNγ mRNA was delayed. The accumulation of TNFα, IL-1β and IFNγ mRNA was paralleled by an increase in the formation of these cytokines in lung and liver.30–32

We further demonstrated that single-dose administration of the ketolide telithromycin as well as the macrolide roxithromycin attenuated the LPS-induced increase in TNFα, IL-1β and IFNγ generation. It has been reported that multiple dose treatment with roxithromycin, orally administered for weeks, decreased the production of proinflammatory cytokines, such as TNFα and IL-1β, in response to LPS in mice.5 Our data show that even a single dose of roxithromycin, intraperitoneally injected, is sufficient to diminish the LPS-induced increase of these cytokines. Because macrolide antibiotics have been shown to be effective in the treatment of inflammatory airway diseases like diffuse panbronchiolitis or chronic sinusitis, previous in vivo studies have been
focused on the anti-inflammatory efficiency of macrolides with regard to the respiratory tract. We have now demonstrated that the macrolide roxithromycin also attenuates the LPS-induced formation of cytokines in other tissues, such as liver, kidney and heart. Furthermore, we showed that the ketolide telithromycin is just as effective as the macrolide roxithromycin in diminishing the LPS-induced tissue formation of TNFα, IL-1β and IFNγ in our in vivo model of acute systemic inflammation. There were no notable differences in lowering cytokine production between roxithromycin and telithromycin. However, we cannot exclude that there may be differences in lag time between macrolides and ketolides because we did not determine cytokine expression over the whole time course. Several studies have been performed to investigate the suppressive effect of macrolide antibiotics on the production of proinflammatory cytokines in vitro. It has been found that macrolides, e.g. roxithromycin or clarithromycin, inhibit the production of inflammatory cytokines such as TNFα and IL-1β in a dose-dependent manner, suggesting a direct rather than an indirect effect of macrolides on the formation of cytokines. Additionally, it has recently been reported that telithromycin attenuates LPS-induced formation of TNFα and IL-1α and inhibits Shiga toxin-stimulated cytokine production in human monocytes.

The release of proinflammatory cytokines leads to a subsequent induction of other mediators of inflammation, such as NOS-II, in affected tissues and cells. Particularly, TNFα, IL-1β and IFNγ are well-known stimuli of NO formation following

Figure 3. Effect of roxithromycin (RXM) or telithromycin (TEL) on LPS-induced TNFα (a), IL-1β (b) and IFNγ (c) mRNA expression in heart, lung, kidney and liver for 1h (TNFα), 2h (IL-1β) or 6 h (IFNγ) after LPS injection, respectively. Real-time PCR data are normalized to β-actin mRNA. Data are mean ± SEM of 5 animals in each group; stars, *P < 0.05 compared to control; †P < 0.05 compared to LPS for 1, 2 or 6 h.

Figure 4. Influence of roxithromycin (RXM) or telithromycin (TEL) on LPS-induced formation of TNFα (a), IL-1β (b) and IFNγ (c) in lung and liver for 2h (TNFα and IL-1β) or 6 h (IFNγ) after LPS challenge. Tissue concentrations are related to total protein assayed. Data are mean ± SEM of 5 animals in each group. Stars, *P < 0.05 compared to control; †P < 0.05 compared to LPS for 2 or 6 h.
increased NOS-II expression.\textsuperscript{18–20} Accordingly, inhibition of cytokine formation may therefore affect the subsequent induction of this enzyme. On this account, we also investigated the effect of telithromycin in comparison to roxithromycin on LPS-induced NOS-II mRNA expression and likewise on the formation of its product NO. Confirming previous observations, we found that LPS increased NOS-II mRNA abundance, which was accompanied by an increased NO generation.\textsuperscript{27,33} Pretreatment with a single dose of the keto derivative of the 14-membered macrolide telithromycin clearly attenuated LPS-induced expression of NOS-II mRNA and, in part, formation of tissue and plasma NO levels just as well as the macrolide roxithromycin. This finding is in line with previous in vivo and in vitro studies showing an inhibitory effect of macrolides on NO generation.\textsuperscript{26–27} Because roxithromycin as well as telithromycin suppressed the production of proinflammatory cytokines like TNFα, IL-1β and IFNγ, the inhibitory effect of roxithromycin and telithromycin on NOS-II induction and NO generation must be due to the reduced formation of proinflammatory cytokines.

It has been suggested that not only 14- and 15-membered macrolide antibiotics, but not 16-membered macrolides like josamycin, have beneficial anti-inflammatory effects in the treatment of diffuse panbronchiolitis.\textsuperscript{13} In agreement with this structural classification we showed that also the keto derivative of the 14-membered macrolide erythromycin, may have clinical anti-inflammatory efficacy. Our data indicate that it is not the cladinose sugar, which is replaced in telithromycin by a keto group, but the 14- and 15-membered macrolactone ring that is crucial for the anti-inflammatory action of macrolides and ketolides. However, the molecular mechanisms by which these compounds perform their inhibitory effects on LPS-induced cytokine and NO formation are not known. There is some evidence that macrolides may suppress the activation of transcription factors, such as nuclear factor kappa B (NFκB) and activator protein-1 (AP-1), in response to inflammatory stimuli leading to a decreased production of the chemokine IL-8 or of the matrix metalloproteinases-2 and -9 \textsuperscript{34–36} Since gene expression of many inflammatory mediators is known to be regulated by NFκB and AP-1,\textsuperscript{37} suppressed activation of these transcription factors may be, at least partly, a possible mechanism of the inhibitory effects of macrolides and ketolides on the induction of proinflammatory cytokines. However, further work should be conducted to investigate the exact molecular mechanisms of these anti-inflammatory effects of macrolide antibiotics.

Taken together, the present study shows that the keto derivative of the 14-membered macrolide telithromycin has anti-inflammatory activity like conventional macrolides, which depends on their ability to prevent the production of proinflammatory cytokines and the subsequent generation of NO. Thus, these data indicate that telithromycin may exert therapeutic effects independently of its antibacterial activity.

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Transparency declarations

There are no commercial or other associations that might pose a conflict of interest.

References


Telithromycin and LPS-induced systemic inflammation


