International Journal of Antimicrobial Agents 48 (2016) 401–408

Contents lists available at ScienceDirect

International Journal of Antimicrobial Agents

journal homepage: www.elsevier.com/locate/ijantimicag

Inhibitors of macrophage infectivity potentiator-like PPIases affect neisserial and chlamydial pathogenicity

Anastasija Reimer [a], Florian Seufert [b], Matthias Weiwad [c], Jutta Ebert [a], Nicole M. Bzdyl [d], Charlene M. Kahler [d], Mitali Sarkar-Tyson [d,e], Ulrike Holzgrabe [b], Thomas Rudel [a], Vera Kozjak-Pavlovic [a,*]

[a] Biocenter, Chair of Microbiology, University of Würzburg, Am Hubland, 97074 Würzburg, Germany
[b] Institute of Pharmacy and Food Chemistry, University of Würzburg, Am Hubland, 97074 Würzburg, Germany
[c] Department of Enzymology, Institute of Biochemistry and Biotechnology, Martin-Luther-University Halle-Wittenberg, Halle, Germany
[d] Marshall Center for Infectious Disease Research and Training, School of Pathology and Laboratory Medicine, University of Western Australia, Perth, WA, Australia
[e] Defence Science and Technology Laboratory, Biomedical Sciences, Porton Down, Salisbury, Wiltshire SP4 0JQ, UK

ARTICLE INFO

Article history:
Received 15 April 2016
Accepted 18 June 2016

Keywords:
Neisseria gonorrhoeae
Neisseria meningitidis
Chlamydia trachomatis
Macrophage infectivity potentiator
Pipecolic acid derivatives

ABSTRACT

The pathogenic bacteria *Chlamydia trachomatis*, *Neisseria gonorrhoeae* and *Neisseria meningitidis* express the surface-exposed macrophage infectivity potentiator (MIP)-like protein, which plays a role in their pathogenicity. MIP exhibits a peptidyl-prolyl isomerase (PPIase) activity that is inhibited by rapamycin and FK506. In this study, pipecolic acid derivatives were tested for their activity against the chlamydial and neisserial MIP. Two MIP inhibitors were identified, PipN3 and PipN4, that affected the developmental cycle of *C. trachomatis* in HeLa cells. Furthermore, we could show that deletion of neisserial MIP or addition of the two MIP inhibitors affected the survival of *N. gonorrhoeae* in the presence of neutrophils. Furthermore, both compounds inhibited the adherence, invasion and/or survival of *N. meningitidis* in epithelial cells. These results confirm the importance of MIP-like proteins in infection and indicate the relevance of pipecolic acid derivatives as antimicrobials against *C. trachomatis*, *N. gonorrhoeae* and *N. meningitidis*.

1. Introduction

Macrophage infectivity potentiator (MIP) has been identified as a virulence-associated protein in various pathogenic bacteria [1,2]. MIP proteins belong to the family of FK506-binding proteins (FKBPs) and exhibit a peptidyl-prolyl *cis*/*trans* isomerase (PPIase) enzyme activity. This activity can be inhibited by the fungal immunosuppressant macrocyclic compounds rapamycin (sirolimus) and FK506 (tacrolimus) [3]. The first characterised MIP was the *Legionella pneumophila* MIP (Lp-MIP), which is involved in initiation of macrophage infection and intracellular survival [4–6]. Subsequently, homologous MIP-like proteins with PPIase activity were identified in pathogens such as *Trypanosoma cruzi*, *Burkholderia pseudomallei*, *Chlamydia trachomatis*, *Neisseria gonorrhoeae* and *Neisseria meningitidis* [7–11].

C. trachomatis, a Gram-negative, obligate intracellular human pathogen, is the most common cause of sexually transmitted infections and infections of the upper inner eyelid (trachoma). The pathogen exhibits a biphasic developmental cycle that takes place in a specialised vacuole called an 'inclusion'. The infection is initiated by infectious and metabolically inert elementary bodies (EBs), which can differentiate inside the inclusion to replicative reticulate bodies (RBs). At the end of the cycle, the RBs differentiate back to EBs that are able to infect new mucosal epithelial cells upon cell lysis. *C. trachomatis* can also transition to a state of persistence upon treatment with, for example, penicillin. The inclusions appear as enlarged, non-dividing and aberrant reticulate bodies (ABs). This persistence can be reversed by changing the inducing factors [12]. The MIP-like protein of *C. trachomatis* (Ctr-MIP) is present on RBs and in the inner and outer membrane of EBs [13,14]. The function of the protein is unknown, but it has been hypothesised that the inner membrane-attached PPIases might catalyse folding of periplasmic or outer membrane proteins that were translocated across the cytoplasmic membrane [1]. In addition, the surface Ctr-MIP can play a role in infection, since treatment of chlamydia with rapamycin prior to or in the course of infection resulted in decreased infectivity [15]. Furthermore, the recombinant Ctr-MIP was shown to induce the production of pro-inflammatory cytokines by monocytes and macrophages [16].

The Gram-negative diplococcus *N. gonorrhoeae* causes the second most prevalent sexually transmitted disease, gonorrhoea. Although treatable, *N. gonorrhoeae* was recently classified as a

* Corresponding author. Fax: +49 931 31 84402.
E-mail address: Vera.Kozjak@uni-wuerzburg.de (V. Kozjak-Pavlovic).

http://dx.doi.org/10.1016/j.ijantimicag.2016.06.020
0924-8579/

'superbug' owing to the rising number of antibiotic-resistant strains and infections [17]. The pathogen causes urethritis in men and cervicitis in women, but can also lead to the development of disseminated gonococcal infections. In symptomatic infections, *N. gonorrhoeae* triggers a potent innate immune response leading to the recruitment of polymorphonuclear leukocytes (PMNs) [18]. Different studies have shown that gonococci are able to evade killing by PMNs and possess different mechanisms that enable them to persist and multiply within neutrophils [19–21]. *N. gonorrhoeae* are also engulfed by macrophages but seem to be able to survive in these immune cells as well [8,22]. The intracellular survival of *N. gonorrhoeae* within macrophages is, however, compromised upon deletion of the *N. gonorrhoeae* MIP-like protein (Ng-MIP). Ng-MIP is a 28.9 kDa lipoprotein localised on the surface of the bacterium [8]. Ng-MIP displays PPIase activity that can be inhibited by rapamycin, similar to Ctr-MIP [8,15].

N. meningitidis, another Gram-negative diplococcus from the genus *Neisseria*, is a commensal of the human nasopharynx, carried asymptomatically in ca. 10% of the adult population in Europe and the USA [23]. Both gonococcus and meningococcus express the highly conserved and surface-exposed MIP-like protein Nm-MIP, which has been shown to be important for bacterial survival in blood [24,25]. Furthermore, the protein has been considered as a vaccine antigen owing to its immunogenicity, its abundance in the outer membrane and its conserved structure [24,26].

The strong connection of MIP-like PPIases to bacterial virulence led to investigation of these proteins as potential drug targets. Pipecolic acid derivatives have been synthesised on the basis of the binding of rapamycin/FK506 to Lp-MIP and *B. pseudomallei* MIP (BpML1) and were tested successfully as MIP inhibitors [11,27,28]. Rapamycin and FK506 by themselves are inappropriate for treatment of infections owing to their immunosuppressive properties. However, the synthesised inhibitors do not exhibit such properties [28]. In this study, we show that pipecolic acid derivatives are also effective inhibitors against Ctr-MIP, Nm-MIP and Ng-MIP. The inhibitory effect of the two compounds on the PPIase activity of recombinant Ctr-Mip and Ng-Mip was demonstrated. The inhibitors negatively affected the chlamydial developmental cycle, thus causing less infectious progeny. These compounds also inhibited the invasion and intracellular survival of *N. meningitidis* in epithelial cells. Furthermore, we showed that Ng-MIP was required for the engulfment and survival of *N. gonorrhoeae* in PMNs. In agreement with this, MIP inhibitors affected the number of viable *N. gonorrhoeae* upon infection of PMNs. These results indicate that inhibitors of MIP-like proteins are important antimicrobial compounds affecting chlamydial growth, survival of *N. gonorrhoeae* in the presence of immune cells, and invasion/survival of *N. meningitidis* in epithelial cells.

2. Materials and methods

2.1. Cell culture, isolation and microscopy

Human epithelial carcinoma cells (HeLa) were cultured in RPMI-1640 (Gibco; Thermo Fisher Scientific, Waltham, MA) medium supplemented with 10% (v/v) heat-inactivated foetal bovine serum (FBS) (Biochrom Ltd., Berlin, Germany) at 37 °C under 5% CO_2. Detroit 562 epithelial cells (immortalised human pharyngeal carcinoma ATCC CCL-138) were grown in Minimum Essential Medium (MEM) with Earle's salt supplemented with 2% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate and 1 × non-essential amino acids (Gibco, Life Technologies Australia, Mulgrave, VIC, Australia). PMNs were isolated from human venous blood using the Ficoll-Hypaque™ method [29]. Transmission electron microscopy (TEM) was performed as previously described [30].

2.2. Bacterial strains and cultivation

C. trachomatis serovar L2 and *C. trachomatis* C2 (L2/434/BU), a green fluorescent protein (GFP)-expressing strain generated essentially as described by Wang et al [31], were stored in SPG buffer (0.22 M sucrose, 10 mM Na_2HPO_4, 3.8 mM K_2HPO_4, 5 mM glutamate, pH 7.4) at –80 °C.

N. gonorrhoeae MS11 derivative strain N2009 ($PorB_{IA}$; Opa-; Pili +; Cam^r; Erm^r) [32] was grown on GC agar plates (Oxoid; Thermo Fisher Scientific) supplemented with 1% vitamin mix for 14–17 h at 37 °C in 5% CO_2. Transformation was performed according to the protocol described in Remmele et al [33].

N. meningitidis strain NMB was grown on GC agar at 37 °C in 5% CO_2 for 14 h prior to infection.

2.3. Generation of Ng-MIP knockout mutant and complementation

The neisserial MIP was deleted by replacing the gene with a kanamycin resistance cassette. For this, a PCR fragment containing the kanamycin sequence flanked by 500 bp upstream and downstream of the *mip* gene was constructed and transformed into *N. gonorrhoeae* strain N2009. The plasmid pLAS::pPilEmCherry (a gift from Berenike Maier), which was used for complementation of the knockout, features an aspartate aminotransferase (NGFG_01468) gene and an L-lactate permease (NGFG_01471) gene. The plasmid was transformed into the MIP deletion strain, resulting in integration of *mip* in the non-coding region between NGFG_01468 and NGFG_01471.

2.4. Protein purification of Ng-MIP and Ctr-MIP and the PPIase assay

The neisserial *mip* gene was amplified by PCR from the genomic DNA of N927 [34] and was cloned without the signal sequence into pET15b (Merck Millipore; Billerica, MA) to introduce an N-terminal 6 × His-tag. Protein expression was induced by 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) (Roth GmbH & Co. KG, Karlsruhe, Germany) for 4 h. The protein was purified using Ni-NTA (QIAGEN, Venlo, The Netherlands) according to the manufacturer's protocol for the isolation of native protein. The His-tag was removed by cleaving with thrombin (Sigma-Aldrich, St Louis, MO). Ctr-MIP and Ng-MIP were further purified on an anion exchange column (Source Q; GE Healthcare, Chicago, IL) and by gel filtration (GE Healthcare).

The PPIase activity of MIP proteins was determined in a coupled assay with the protease α-chymotrypsin [35].

2.5. Neutrophil survival assay

Isolated PMNs (2×10^5 cells) were seeded in 24-well plates (Corning Inc., Corning, NY) by centrifuging the cells down to the plate bottom at 1000 rpm for 5 min. The cells were infected in RPMI medium without FBS at a multiplicity of infection of 50 with *N. gonorrhoeae* for 1 h at 37 °C under 5% CO_2. The cells were washed three times with medium to remove extracellular bacteria and were incubated for a further 1–2 h. Afterwards, the cells were washed three times and were lysed with 100 µL of 1% saponin (Sigma) for 7 min. Then, 400 µL of fresh medium was added to each well and the bacteria were plated at dilutions of 10^{-1}, 10^{-2} and 10^{-3} on GC agar plates. Following overnight incubation at 37 °C, the grown colonies were counted by using a binocular microscope (Motic, Hong Kong, China).

2.6. Bioactivity screening and progeny assay

The bioactivity screening and progeny assay were performed as previously described by Reimer et al [36]. The protocol of the progeny assay in this study differed only regarding the analysis time of the