The prenylation inhibitor manumycin A reduces the viability of *Anaplasma phagocytophilum*

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Anaplasma phagocytophilum is an obligately intracellular bacterium and is the causative agent of human granulocytic anaplasmosis (HGA), an emerging and major tick-borne disease in the USA and other parts of the world. This study showed that the prenylation inhibitor manumycin A effectively blocked *A. phagocytophilum* infection in host cells (HL-60 or RF/6A cells).

A. phagocytophilum infection activated extracellular signal-regulated kinase (ERK) mitogen-activated protein kinase in host cells, and manumycin A treatment reduced ERK activation in *A. phagocytophilum*-infected host cells. As ERK activation is required for *A. phagocytophilum* infection, we examined whether manumycin A inhibited the bacteria directly or through host ERK signalling. Treatment of *A. phagocytophilum* alone with manumycin A significantly reduced the bacterial infectivity of host cells and bacterial viability in the absence of host cells, whereas pre-treatment of host cells did not inhibit bacterial infection in host cells. The inhibitory effect of manumycin A on *A. phagocytophilum* infection in host cells was achieved even at a concentration 100 times lower than that required for effective inhibition of mammalian cell signalling. These results suggested that manumycin A directly inactivates the bacterium, resulting in reduced infection and ERK1/2 activation. Thus, the manumycin group of drugs may have a therapeutic potential for HGA.

Received 18 December 2010

Accepted 23 February 2011

INTRODUCTION

Anaplasma phagocytophilum is a tick-borne Gram-negative bacterium that proliferates in membrane-bound inclusions in granulocytes and endothelial cells of various mammalian species (Dumler *et al.*, 2001; Goodman *et al.*, 1996; Munderloh *et al.*, 2004). In humans, *A. phagocytophilum* causes human granulocytic anaplasmosis (HGA), an emerging and major tick-borne disease in the USA and other parts of the world. Since it was first identified in the early 1990s in a Wisconsin patient who died from a severe febrile illness after a tick bite (Chen *et al.*, 1994), HGA has been increasingly recognized in North America, Europe and Asia as a cause of an acute and severe febrile disease after a tick bite (Bakken & Dumler, 2008; Parkins *et al.*, 2009; Zhang *et al.*, 2008). HGA is often accompanied by headache, myalgia and malaise. Severe complications include prolonged fever, shock, confusion, acute renal failure, haemorrhage, rhabdomyolysis, opportunistic infection by viral and fungal agents, and even death when diagnosis and treatment are delayed (Bakken & Dumler, 2008). The outcome of HGA is poor in elderly or immunocompromised individuals (Bakken & Dumler,

2000). The number of reported anaplasmosis cases has increased every year since this disease was selected as one that should be notified to the Centers for Disease Control and Prevention in 1999. Compared with 2007, the number of cases attributed to *A. phagocytophilum* in 2008 had increased by 21 % (Hall-Baker *et al.*, 2010). In the recent summary of notifiable diseases in the USA during 2009, 1161 cases of HGA were reported (CDC, 2010).

Because of the potential for severe and fatal disease, chemotherapy is indicated for all suspected or confirmed HGA cases. The drug of choice is a tetracycline antibiotic, usually doxycycline, as *A. phagocytophilum* is highly susceptible to doxycycline. If there is no significant treatment delay, the response to doxycycline is usually very rapid, with improvement evident within 24–48 h (Bakken & Dumler, 2008). Although tetracycline-based drugs are generally effective in treating *A. phagocytophilum* infection, therapies for children and pregnant women are still urgently needed because of the side effects of tetracycline (Korzeniowski, 1995; Lochary *et al.*, 1998). Rifampicin and the new fluoroquinolone antibiotic trovafloxacin are effective *in vitro* (Horowitz *et al.*, 2001; Klein *et al.*, 1997), but limited *in vivo* studies are available to confirm the *in vitro* data (Buitrago *et al.*, 1998; Krause *et al.*, 2003). Therefore, rifampicin is recommended only for patients with contraindications to doxycycline or tetracycline therapy (Bakken & Dumler,

Abbreviations: ERK, extracellular signal-regulated kinase; HGA, human granulocytic anaplasmosis; IC₅₀, 50 % inhibition concentration; p.i., post-inoculation.

2008). Currently, no other therapeutic drugs are available for HGA treatment.

A. phagocytophilum resides and proliferates in the professional killer cells of the host and disrupts many cell functions, in particular by modulating host-cell signalling to benefit itself and establish infection in the host (Rikihisa, 2003, 2006). It has been shown that *A. phagocytophilum* activates extracellular signal-regulated kinase (ERK) in neutrophils and HL-60 cells (Lee *et al.*, 2008; Sukumaran *et al.*, 2011; Xiong *et al.*, 2009) and that ERK activation is required for *A. phagocytophilum* infection (Xiong *et al.*, 2009). One of the best-characterized ERK activation pathways is Ras/Raf/MEK/ERK (Roux & Blenis, 2004), and manumycin A, a farnesyltransferase inhibitor, is known to inhibit this pathway by inhibiting Ras farnesylation (Ito *et al.*, 1996). The aim of this study was to determine the effects of manumycin A on the growth of *A. phagocytophilum* in mammalian host cells and the potential mechanism of this inhibitory effect.

METHODS

Reagents and antibodies. Manumycin A was purchased from Calbiochem. Mouse mAb 5C11, which recognizes the N-terminal

conserved region of the *A. phagocytophilum* major surface protein P44, has been described previously (Kim & Rikihisa, 1998). Other antibodies used were mouse mAb against phospho-ERK1/2 (E10; Cell Signaling), rabbit anti-ERK1/2 (Cell Signaling) and mouse mAb against α -tubulin (Santa Cruz Biotechnology). Peroxidase-conjugated secondary antibodies were obtained from KPL.

***A. phagocytophilum* culture and manumycin A treatments.** The *A. phagocytophilum* HZ strain was cultivated in the human promyelocytic leukaemia cell line HL-60 (Rikihisa *et al.*, 1997). Host-cell-free *A. phagocytophilum* was prepared by sonicating highly infected (>90 % infected) HL-60 cells for 8 s twice at an output setting of 2 with an ultrasonic processor (W-380; Heat Systems). After low-speed centrifugation at 500 g for 5 min to remove nuclei and unbroken cells, the supernatant was centrifuged at 10 000 g for 10 min and the pellet, which was enriched with host-cell-free organisms, was added to HL-60 or endothelial RF/6A cells. After 2 h incubation at 37 °C, extracellular organisms were washed, fresh medium was added and this time point was considered 0 h post-inoculation (p.i.). Cells continued to be incubated at 37 °C.

Manumycin A was added before infection (pre-treatment) or at indicated time points (0 h or 1 day p.i.) and the inhibitor was kept in the growth medium throughout the incubation period or was removed later as indicated. Inhibitor treatments at these concentrations did not affect host-cell integrity as assessed by light microscopy and trypan blue staining. The degree of bacterial infection in host cells was assessed by Diff-Quik staining (Baxter Scientific Products) and

Fig. 1. Manumycin A blocks *A. phagocytophilum* infection in host cells. Manumycin A (0, 0.01, 0.1 and 1 μM in 1 % DMSO) was added to HL-60 cells (a, b) or RF/6A cells (c) at 0 h p.i. (a, c) or 1 day p.i. (b, d). The number of bacteria was determined at 2 days p.i. (a, b) or 3 days p.i. (c). Data are expressed as means $\pm$ SD ($n=3$) and are representative of three independent experiments with similar results. *, $P<0.05$; **, $P<0.01$ (unpaired two-tailed t -tests). (d) At 2 days p.i., infected cells treated with 1 μM manumycin A and vehicle-control DMSO were harvested and observed by light microscopy following Diff-Quik staining. Arrows indicate *A. phagocytophilum* inclusions. Note the much smaller inclusions, which contained fewer bacteria, in the manumycin A-treated cell compared with the control DMSO-treated cell. Bar, 5 μm.

the number of *A. phagocytophilum* cells was estimated in 200 host cells in triplicate culture wells (Rikihisa *et al.*, 1995).

Western blot analysis. *A. phagocytophilum*-infected and uninfected HL-60 cells (1×10^6) were washed, resuspended in 200 μ l ice-cold RIPA buffer (50 mM Tris/HCl, pH 7.4; 1 % NP-40; 0.25 % sodium deoxycholate; 150 mM NaCl; 1 mM EDTA) containing freshly added Protease Inhibitor Cocktail Set III and Phosphatase Inhibitor Cocktail Set II (Calbiochem) and lysed by mixing with 200 μ l 2 $\times$ Laemmli sample buffer (135 mM Tris/HCl, pH 6.8; 4 % SDS; 20 % glycerol; 10 % β -mercaptoethanol). Samples were separated by SDS-PAGE on 7.5 or 10 % polyacrylamide gels and then transferred to a nitrocellulose membrane with a semi-dry blotter (WEP). The membrane was blocked with 5 % (w/v) skimmed milk (BD) in Tris-buffered saline (50 mM Tris/HCl, pH 7.5; 150 mM NaCl) containing 0.1 % Tween 20, incubated with primary antibodies (diluted 1:500 or 1:1000) at 4 °C for 12 h and subsequently incubated with peroxidase-conjugated secondary antibodies (diluted 1:1000) at room temperature for 1 h. Immunoreactive bands were visualized with enhanced chemiluminescence (Thermo Scientific) using a CCD camera (Fuji LAS-3000 Imaging System) and band density was measured using Fujifilm MultiGauge software.

Immunofluorescence assay and live/dead bacterial staining. Host-cell-free *A. phagocytophilum* was filtered through a 2.7 μ m filter and pre-treated with manumycin A (1 μ M) for 15 min in SPK buffer (0.05 M potassium phosphate, pH 7.4; 0.2 M sucrose), followed by staining for 15 min with a LIVE/DEAD BacLight Bacterial Viability kit (Molecular Probes, Invitrogen). Cells were then mounted on slides and observed under a Nikon Eclipse E400 fluorescence microscope with a xenon/mercury light source (Nikon Instruments).

Statistical analysis. Statistical analyses were performed with unpaired, two-tailed Student's *t*-tests using Microsoft Office Excel. A value of $P < 0.05$ was considered to be significant.

RESULTS

Manumycin A blocks *A. phagocytophilum* infection in HL-60 and RF/6A cells

We and others have shown previously that ERK signalling is highly activated in *A. phagocytophilum*-infected HL-60 cells and neutrophils (Lee *et al.*, 2008; Xiong *et al.*, 2009).

Because Ras/Raf/ERK are components of the major canonical mitogen-activated protein kinase activation pathway, we used the Ras prenylation inhibitor manumycin A to determine whether Ras is upstream of the cascade of ERK signalling induced by *A. phagocytophilum* infection in host cells. Manumycin A effectively blocked *A. phagocytophilum* infection in HL-60 cells in a dose-dependent manner when manumycin A was added to the cell culture at 0 h p.i. (Fig. 1a). Significant inhibition was achieved at a concentration as low as 0.01 μ M (Fig. 1a). In addition, the inhibitory effect of manumycin A was still observed if the drug was added to the cell culture at 1 day p.i. (around 40 % infected) (Fig. 1b). The bacteria in HL-60 cells were cleared by manumycin A treatment at a dosage of 1 μ M. The *A. phagocytophilum* inclusions became smaller and looser in manumycin A-treated HL-60 cells (Fig. 1d). Similar experiments were carried out in the endothelial cell line RF/6A, which can be infected with *A. phagocytophilum* (Munderloh *et al.*, 2004), and the same results were found (Fig. 1c).

Manumycin A blocks ERK activation induced by *A. phagocytophilum* infection in HL-60 cells

Because our previous data showed that ERK activation is required for *A. phagocytophilum* infection (Xiong *et al.*, 2009), we examined whether the reduced *A. phagocytophilum* infection was due to abolished ERK activation by manumycin A. Manumycin A blocked *A. phagocytophilum* infection (indicated by the relative protein levels of the *A. phagocytophilum* major surface protein P44) as well as ERK activation (indicated by the relative levels of phosphorylated ERK1/2) in HL-60 cells (Fig. 2).

Pre-treatment of *A. phagocytophilum* with manumycin A abolishes *A. phagocytophilum* infection in HL-60 cells

Manumycin A is a potent and selective eukaryotic farnesyl-transferase inhibitor with a 50 % inhibition concentration

Fig. 2. *A. phagocytophilum* infection activates ERK and manumycin A inhibits both bacterial infection and ERK activation in infected HL-60 cells. Western blot analysis was performed with antibodies specific to phosphorylated ERK (p-ERK), total ERK1/2 and P44, the *A. phagocytophilum* major surface protein. Uninfected and *A. phagocytophilum*-infected HL-60 cells (Ap) with or without manumycin A treatment were collected at 3 days p.i. α -Tubulin was used as a protein loading control to normalize each sample. The values under the bands show the ratios of band intensities of P44, p-ERK1/2 and total ERK (T-ERK) to α -tubulin, respectively. Data are representative of three independent experiments.

Fig. 3. Pre-treatment of *A. phagocytophilum* alone with manumycin A inhibits bacterial infection in host cells. (a) Host-cell-free *A. phagocytophilum* was pre-treated with serially diluted manumycin A for 15 min at 37 °C in SPK buffer and, after washing, was inoculated onto non-treated HL-60 cells. **, $P < 0.01$ (unpaired two-tailed t -test). (b) Host HL-60 cells were pre-treated with serially diluted manumycin A in RPMI for 15 min at 37 °C. After washing, they were inoculated with non-treated *A. phagocytophilum*. The number of bacteria was determined at 2 days p.i. (c) Host-cell-free *A. phagocytophilum* was pre-treated with 1 μM manumycin A or 1 % DMSO (control) for different time periods (2, 10 and 30 min) at 37 °C in SPK buffer. A time of 0 min indicates cells that were not exposed to manumycin A or DMSO. After washing, the cells were inoculated onto HL-60 cells. The number of bacteria was determined at 2 days p.i. Data are expressed as means $\pm$ SD ($n=3$) and are representative of three independent experiments with similar results. *, $P < 0.05$; **, $P < 0.01$ (unpaired two-tailed t -tests). P values were calculated for manumycin A-treated versus vehicle control for each time point.

(IC_{50}) of 5 μM (Hara *et al.*, 1993), which is much higher than the concentration (<0.1 μM) used in our study to block *A. phagocytophilum* growth. This discrepancy led us to speculate that the reduced *A. phagocytophilum* infection in host cells may be due to manumycin A targeting the bacterium itself. To test this possibility, we pre-treated *A. phagocytophilum* with manumycin A and then infected host cells after removal of the drug by washing. Treatment of

A. phagocytophilum with manumycin A significantly accelerated the loss of infectivity in a dose- and time-dependent manner (Fig. 3a, c). In addition, this inactivation of *A. phagocytophilum* was highly effective, as only 2 min of treatment with manumycin A (1 μM) resulted in a significant reduction in bacterial infectivity (Fig. 3c). In contrast, the infectivity of *A. phagocytophilum* was not different when host HL-60 cells were pre-treated with manumycin A (Fig. 3b).

Fig. 4. Manumycin A reduces isolated *A. phagocytophilum* viability. Host-cell-free *A. phagocytophilum* was pre-treated with 1 μM manumycin A or 1 % DMSO for 15 min in SPK buffer, followed by staining for 15 min with the LIVE/DEAD BacLight Bacterial Viability kit, and was observed by fluorescence microscopy. A green signal indicates viable bacteria and a red signal indicates dead bacteria. Data are representative of three independent experiments with similar results. Bars, 5 μm.

Manumycin A reduces *A. phagocytophilum* viability in the absence of host cells

Because *A. phagocytophilum* pre-treatment with manumycin A caused a significant reduction in bacterial growth in host cells, we examined bacterial viability after manumycin A treatment with the LIVE/DEAD BacLight Bacterial Viability kit. Over 200 total green (live) or red (dead) bacteria from different randomly selected fields as shown in Fig. 4 were scored. The percentage of live *A. phagocytophilum* in the manumycin A-treated group was $24.3 \pm 2.0\%$, which is significantly lower than $78.2 \pm 2.1\%$ in the control group ($n=3$, $P < 0.01$).

DISCUSSION

In this study, we demonstrated that *A. phagocytophilum* infection in host cells was effectively blocked by manumycin A and that this inhibitory effect was due to the antimicrobial effect of manumycin A on the bacteria. To our knowledge, this is the first report of biological activity of manumycin against Gram-negative bacteria.

We showed previously that the cholesterol biosynthesis inhibitor lovastatin does not block but rather enhances *A. phagocytophilum* growth in culture (Xiong *et al.*, 2009). Because both cholesterol and farnesylated-pyrophosphate are derived from the mevalonate pathway, lovastatin not only blocks *de novo* cholesterol synthesis but also blocks the synthesis of farnesylated-pyrophosphate, which is used in Ras prenylation on the membrane (Murthy *et al.*, 2005). Our *in vitro* lovastatin data suggested that Ras prenylation may not be involved in *A. phagocytophilum* infection. This was puzzling at first for us, as the Ras farnesylation inhibitor manumycin A had strong inhibitory effects on ERK activated by *A. phagocytophilum* infection. Our current data showing that manumycin A has a direct effect on *A. phagocytophilum* solved this conundrum. Therefore, *A. phagocytophilum* infection may activate ERK in an alternative pathway. Recently, an *A. phagocytophilum* virulence protein, AptA, was identified as being involved in the activation of ERK1/2 during *A. phagocytophilum* infection (Sukumaran *et al.*, 2011).

Manumycin A has been tested as an anti-cancer treatment in thyroid carcinoma, but concern was raised because of its toxicity *in vitro* and in animal models (Xu *et al.*, 2001; Yeung *et al.*, 2000). The IC_{50} of manumycin against yeast farnesyltransferase is $5 \mu\text{M}$ (Hara *et al.*, 1993). Manumycin A was shown to reduce the viability of all six human anaplastic thyroid cancer cell lines tested at concentrations of over $5 \mu\text{M}$; however, manumycin A also inhibited the proliferation and viability of endothelial cells at concentrations of $2 \mu\text{M}$ (Xu *et al.*, 2001; Yeung *et al.*, 2000). Below $1 \mu\text{M}$, manumycin A has no inhibitory effects on mammalian cells (Xu *et al.*, 2001; Yeung *et al.*, 2000). The optimal concentration of manumycin A found in our study to block *A. phagocytophilum* infection in host cells was $0.1 \mu\text{M}$, and in fact the inhibitory effect could be achieved at a

concentration as low as $0.01 \mu\text{M}$, which is 100 times lower than the concentration used for anti-cancer studies. This difference indicates that *A. phagocytophilum* is highly sensitive to manumycin A treatment and this inhibitory effect is not dependent on inhibition of host-cell signalling.

There are only a few reports about the antimicrobial activity of manumycin A. Because manumycin A has anti-Gram-positive bacterial activity *in vitro*, manumycin class antibiotics including U-62162 (8 mg kg^{-1}) and U-56,407 (320 mg kg^{-1}) have been tested in mice that were experimentally infected with *Staphylococcus aureus* (Brodasky *et al.*, 1983; Slechta *et al.*, 1982). Ali *et al.* (1999) reported that manumycin A is a sensitive and novel trypanocide *in vitro* (IC_{50} values against two *Trypanosoma brucei* strains, BSF and PCF, were 1.5 and $0.4 \mu\text{M}$, respectively), because it alters protein prenylation in *T. brucei* and causes mitochondrial damage. The biosynthesis of manumycin group compounds has been studied intensively and new analogues can be produced by manipulating the biosynthetic pathways such as by feeding artificial precursors or changing the cultivation parameters by increasing oxygen partial pressure (Sattler *et al.*, 1998). Thus, in light of the extremely high sensitivity of *A. phagocytophilum* to manumycin A, it is worth testing these new manumycin derivatives or other farnesyltransferase inhibitors for *A. phagocytophilum* inhibition *in vitro* and *in vivo* in the future. *A. phagocytophilum* has diverse host animal specificity, several genes are known to be variable among strains and potentially cell culture passage numbers may alter bacterial virulence (Dumler *et al.*, 2001; Pusterla *et al.*, 2000; Rikihisa *et al.*, 2010; Scharf *et al.*, 2011); therefore, it will also be prudent to examine these variable factors in the future.

ACKNOWLEDGEMENTS

This research was supported by NIH grant R01 AI30010.

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