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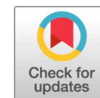

Orientia tsutsugamushi Is Highly Susceptible to the RNA Polymerase Switch Region Inhibitor Corallopyronin A *In Vitro* and *In Vivo*

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ABSTRACT Scrub typhus is a potentially lethal infection caused by the obligate intracellular bacterium *Orientia tsutsugamushi*. Reports on the emergence of doxycycline-resistant strains highlight the urgent need to develop novel antiinfectives against scrub typhus. Corallopyronin A (CorA) is a novel α-pyrone compound synthesized by the myxobacterium *Corallococcus coralloides* that was characterized as a noncompetitive inhibitor of the switch region of the bacterial RNA polymerase (RNAP). We investigated the antimicrobial action of CorA against the human-pathogenic Karp strain of *O. tsutsugamushi in vitro* and *in vivo*. The MIC of CorA against *O. tsutsugamushi* was remarkably low (0.0078 μg/ml), 16-fold lower than that against *Rickettsia typhi*. In the lethal intraperitoneal *O. tsutsugamushi* mouse infection model, a minimum daily dose of 100 μg CorA protected 100% of infected mice. Two days of treatment were sufficient to confer protection. In contrast to BALB/c mice, SCID mice succumbed to the infection despite treatment with CorA or tetracycline, suggesting that antimicrobial treatment required synergistic action of the adaptive immune response. Similar to tetracycline, CorA did not prevent latent infection of *O. tsutsugamushi in vivo*. However, latency was not caused by acquisition of antimicrobial resistance, since *O. tsutsugamushi* reisolated from latently infected BALB/c mice remained fully susceptible to CorA. No mutations were found in the CorA-binding regions of the β and β' RNAP subunit genes *rpoB* and *rpoC*. Inhibition of the RNAP switch region of *O. tsutsugamushi* by CorA is therefore a novel and highly potent target for antimicrobial therapy for scrub typhus.

KEYWORDS *Orientia tsutsugamushi*, RNA polymerase inhibitors, corallopyronin A, myxobacteria, rickettsiosis, scrub typhus, vector-borne infection

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Scrub typhus, the disease caused by *Orientia tsutsugamushi*, is a potentially fatal mite-borne infection that is endemic in the Asia-Pacific region. Worldwide, more than 1 billion people are estimated to be at risk to acquire the infection (1). Recent reports from other continents suggest that the global distribution of *Orientia* species may have been largely underestimated (2–5). Despite its medical importance, only a limited set of drug targets is available for therapeutic use. Treatment strategies currently encompass two approaches: i) bacterial ribosomal protein biosynthesis can be inhibited by tetracyclines (e.g., doxycycline), macrolides (e.g., azithromycin), or chloramphenicol, and ii) RNA transcription by the bacterial RNA polymerase (RNAP) can be inhibited by rifamycins (e.g., rifampin). Although it has not been investigated if resistance to rifampin occurs in *O. tsutsugamushi*, rifampin resistance can be experimentally induced in typhus group rickettsiae (6, 7) and was found in naturally occurring strains of spotted fever group rickettsiae (8), both close relatives of *O. tsutsugamushi*. More importantly, reports on doxycycline-resistant scrub typhus in regions where it is highly endemic emphasize the urgent need to identify novel therapeutic drug targets in *O. tsutsugamushi* (9, 10).

In the development of novel antimicrobials, inhibition of bacterial RNAP is regarded as a particularly promising approach. However, only two groups of RNAP inhibitors are clinically available for the treatment of bacterial infections. Besides rifamycins, which have an indispensable role in tuberculosis therapy (11), fidaxomicin has been developed for clinical use and is nowadays used to treat *Clostridium difficile* infections (12). While rifampin is an established therapy in scrub typhus, the activity of fidaxomicin against *O. tsutsugamushi* is unknown. Recent work has revealed the RNAP switch region as a novel antimicrobial drug target. RNA transcription requires opening and closing of the RNAP β' subunit clamp that swings on the base of the refolding switch region (13, 14). This movement can be blocked allosterically by compounds from the group of α-pyrone antibiotics (corallopyronin and myxopyronin) (13, 14), ripostatin (13), squaramides (15), and others.

It was the aim of the present study to investigate whether corallopyronin A (CorA), a myxobacterial natural product for which the biosynthetic pathway is known (16) and that binds in this switch region (13, 14), could be a potential antimicrobial agent against *O. tsutsugamushi*. CorA was first isolated from the myxobacterium *Corallococcus coralloides* in the 1980s (17, 18) and was shown to inhibit RNA transcription in Gram-positive bacteria (18). Binding of CorA (and myxopyronin) causes refolding of the highly conserved switch 2 segment, which results in a distorted, inactive promoter complex (14). Consequently, CorA locks the RNAP clamp, preventing the entry of double-stranded DNA templates into the enzyme (hinge jamming) (13). The CorA binding determinant encompasses a significant fraction of the RNAP switch region, including switch 2 and adjacent segments of the β and β' subunits (13, 19). Importantly, this binding site is remote from the binding site of rifamycins (13), which prevents the development of cross-resistance.

Low bacterial membrane permeability for antimicrobial compounds (20) or the presence of efflux pumps (21) are frequently encountered obstacles during drug validation. Yet, the particularly lipophilic properties of CorA (18) and its *in vivo* efficacy against the filarial endobacterium *Wolbachia* (22) suggested that CorA was likely to have an antimicrobial effect against other intracellular bacteria. We therefore conducted the present study to investigate the effect of CorA on the human-pathogenic Karp strain of *O. tsutsugamushi* both *in vitro* and in experimental mouse infections.

RESULTS

***O. tsutsugamushi* is highly susceptible *in vitro* to CorA.** To investigate whether CorA is able to inhibit growth *in vitro* of *O. tsutsugamushi* Karp, L929 cells seeded on glass coverslips were infected with *O. tsutsugamushi*, washed, and grown in the presence of 0.125 μg/ml CorA, 10 μg/ml tetracycline, or unsupplemented medium for 4 days. Fluorescence microscopy of 16S fluorescence *in situ* hybridization (FISH)-

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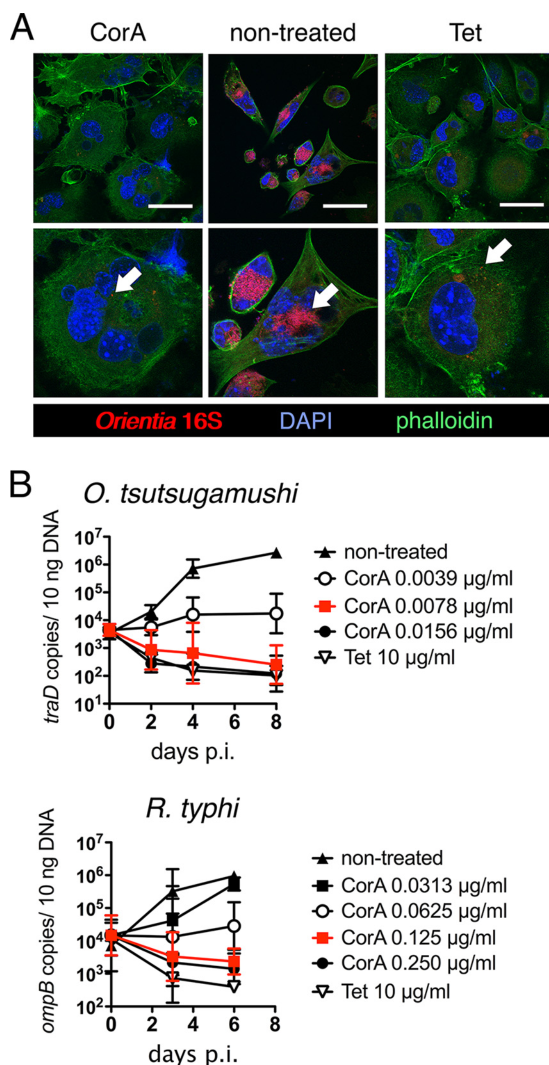

FIG 1 *O. tsutsugamushi* is susceptible to low doses of CorA *in vitro*. (A) 0.75 × 10^5 irradiated L929 cells were grown on coverslips in 24-well plates, infected with *O. tsutsugamushi* Karp, and treated as indicated. On day 4 p.i., cells treated with 10 μg/ml tetracycline, 0.125 μg/ml CorA, or medium were fixed in 4% paraformaldehyde (PFA). *O. tsutsugamushi* was labeled by FISH using the Atto590-labeled oligonucleotide 16S probe *Sitobion miscanthi* L type symbiont (SMLS), followed by fixation with 4% PFA. Actin fibers were stained with Alexa-488-conjugated phalloidin, followed by DAPI (4′,6-diamidino-2-phenylindole) stain. Images were recorded by confocal microscopy (bar, 50 μm). Arrows indicate *O. tsutsugamushi* organisms. (B) 2 × 10^5 irradiated L929 cells were grown in 24-well plates, infected with *O. tsutsugamushi* or *R. typhi*, and treated with 2-fold serial dilutions of CorA at the indicated concentrations, with 10 μg/ml tetracycline, or left untreated (in triplicates). Bacterial loads in infected cell cultures were measured by *traD* qPCR (*O. tsutsugamushi*, upper panel) or *ompB* qPCR (*R. typhi*, lower panel) immediately p.i. and on the indicated days p.i. The MIC is depicted in red. Shown are combined results from two independent experiments (mean ± standard deviation [SD]).

phalloidin-costained cultures showed that CorA inhibited growth of *O. tsutsugamushi* to a similar degree as the tetracycline control (Fig. 1A).

To determine the MIC of CorA for *O. tsutsugamushi*, *traD* copy numbers were measured repeatedly by real-time PCR (23) from *O. tsutsugamushi* cell cultures, which was performed in the presence of 2-fold serial dilutions of CorA. For *O. tsutsugamushi*, an MIC of 0.0078 μg/ml CorA was found (Fig. 1B, upper panel). We repeated a similar experiment with *R. typhi*, which revealed an MIC of 0.125 μg/ml (Fig. 1B, lower panel). Thus, CorA was able to inhibit growth of *O. tsutsugamushi* at an MIC that was 16-fold lower than that for *R. typhi*.

CorA protects against lethal *O. tsutsugamushi* infection *in vivo*. Next, we studied whether CorA is effective *in vivo* against *O. tsutsugamushi*. BALB/c mice were infected

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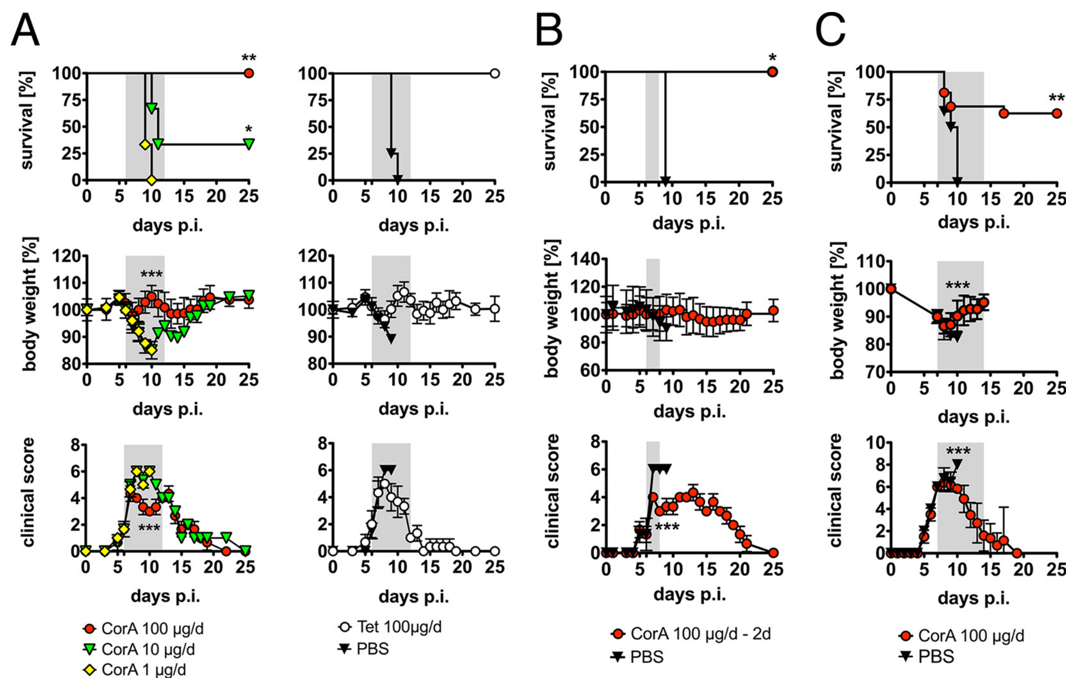

FIG 2 CorA protects BALB/c mice from lethal intraperitoneal *O. tsutsugamushi* infection. BALB/c mice were infected i.p. with *O. tsutsugamushi* Karp (5,000 SFU). (A) Mice were treated with a daily dose of 1 to 100 μg CorA, 100 μg tetracycline, or PBS for up to 7 days, starting at day 6 p.i. Gray areas depict the treatment period. Shown are survival curves (upper panels), change in body weight (middle panels), and combined clinical scores (lower panels; $n = 3$ mice per group). (B) Mice were treated for 2 days with a daily dose of 100 μg CorA or PBS, starting at day 6 p.i. ($n = 3$ mice per group). (C) Infected mice were treated for up to 7 days with a daily dose of 100 μg CorA or PBS, starting at day 7 p.i. Shown are pooled data from two independent experiments ($n = 14$ to 16 mice per group). (A to C) Means ± SD. Survival curves were analyzed by a log-rank (Mantle-Cox) test comparing CorA-treated to PBS-treated groups. Body weight and score data were analyzed by 2-way ANOVA and Bonferroni's *post hoc* test. Shown are significance levels when comparing the 100 μg/day and 10 μg/day groups on day 10 p.i. (A), or CorA- and PBS-treated groups on day 9 (B) or 10 (C) p.i. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

intraperitoneally (i.p.) with the human-pathogenic Karp strain (23, 24). In order to obtain the minimum dose required, BALB/c mice infected i.p. with 5,000 spot-forming units (SFU) received CorA in decreasing doses (10-fold dilutions, from 100 μg to 1 μg), 100 μg tetracycline, or phosphate-buffered saline (PBS) daily for 7 days. Treatment was administered by i.p. injection and initiated at day 6 postinfection (p.i.), 1 day after the onset of symptoms on day 5 p.i. as indicated by an increased clinical score (Fig. 2A). As shown in Fig. 2A, 100 μg/day of CorA protected 100% of treated mice from lethal infection and body weight loss, while 10 μg/day CorA only protected one-third of the mice. Lower concentrations were ineffective, and all PBS-treated control mice succumbed to the infection by day 10 p.i. Thus, a daily dose of 100 μg CorA, which in mice with a starting weight of 18.31 ± 1.09 g corresponds to 5.48 ± 0.35 mg/kg (mean ± standard deviation [SD]), was sufficient to protect from otherwise lethal infection.

In resource-poor settings, a short and effective therapy regimen is particularly important. We therefore investigated whether treatment duration could be shortened and treated the mice for 7, 6, 4, and 2 days. A 2-day course of CorA was sufficient to protect from lethal infection (Fig. 2B) and body weight loss and to induce clearance of symptoms.

In order to test if CorA is able to protect when applied at an even more advanced stage of infection, we delayed the initiation of treatment with CorA, tetracycline, or PBS until day 7 p.i., i.e., after 2 symptomatic days, and continued the application for up to 7 days. In these experiments, 10/16 (60%) CorA- or tetracycline-treated mice survived the infection, compared to 0/12 (0%) PBS-treated control mice (Fig. 2C). Thus, 100 μg/day CorA rescued infected mice from lethal disease courses even at an advanced infection stage.

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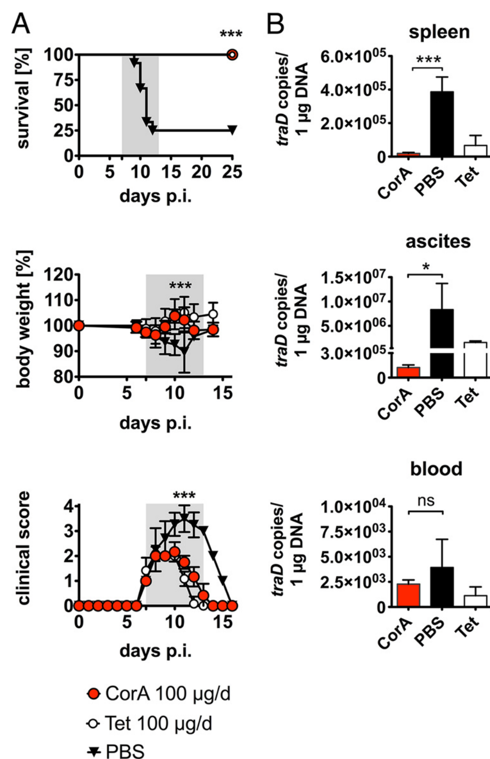

FIG 3 CorA inhibits *O. tsutsugamushi* replication *in vivo*. BALB/c mice were infected i.p. with *O. tsutsugamushi* Karp (1,000 SFU) and treated daily for a course of 7 days with 100 μg CorA, 100 μg tetracycline, or PBS i.p., starting at day 7 p.i. (A) The gray area depicts the treatment period for survival curves (upper panel), body weight changes (middle panel), and clinical scores (lower panel). Shown are combined data from two independent experiments ($n = 12$ mice per group, means $\pm$ SD). ***, $P < 0.001$ by log-rank (Mantle-Cox) test (survival curves) or by 2-way ANOVA with Bonferroni's *post hoc* test (significance level refers to days 9 to 11 in weight charts and days 9 to 15 in score charts). (B) Mice infected with 1,000 SFU *O. tsutsugamushi* i.p. and treated daily with 100 μg CorA, 100 μg tetracycline, or PBS i.p. for 2 days (day 7 and 8) were euthanized on day 9 p.i. Bacterial loads from DNA extracts of spleen, ascites, and blood were measured by *traD* qPCR. Data are from one of two representative experiments ($n = 3$ or 4 per group). Mean $\pm$ SD; *, $P < 0.05$; ***, $P < 0.001$ by one-way ANOVA and Bonferroni's *post hoc* test.

The fulminant course of high-dose i.p. infection in BALB/c mice narrows the therapeutic window in this model to 1 to 2 days after the onset of symptoms. In order to obtain a more protracted development of disease symptoms, we reduced the infection dose to 1,000 SFU. Daily treatment with 100 μg CorA, 100 μg tetracycline, or PBS was started on day 7 p.i. CorA protected all infected mice from lethal outcome, equivalent to tetracycline, while 3/12 (25%) PBS-treated control mice survived the infection (Fig. 3A, upper panels). In comparison to control mice, improvements of body weight and clinical scores in CorA-treated mice could be observed after 2 days of treatment on day 9 p.i. (Fig. 3A, middle and lower panels). To study whether CorA really reduces bacterial replication *in vivo*, we quantified bacterial DNA in mouse organs in CorA-treated and control mice on day 9 p.i., i.e., after 2 days of treatment. Bacterial DNA concentrations in spleen and ascites, but not in blood, were significantly lower from mice that had received CorA compared to in PBS-treated controls (Fig. 3B).

***O. tsutsugamushi* remains susceptible to CorA after entering latency.** It has been shown that latent infection with *O. tsutsugamushi* is established in humans and mice not only after immune-mediated clearance (25–27), but also after treatment with antimicrobials (28). To test whether CorA prevents latency, we used cyclophosphamide to immunosuppress mice that were intradermally (i.d.) infected with *O. tsutsugamushi* (Fig. 4A) at days 54 and 57 p.i. and measured bacterial loads in spleens and lungs. *O. tsutsugamushi* was reactivated, regardless of whether mice had been treated with CorA, tetracycline, or PBS (Fig. 4B). Thus, treatment with CorA, similar to that with tetracycline,

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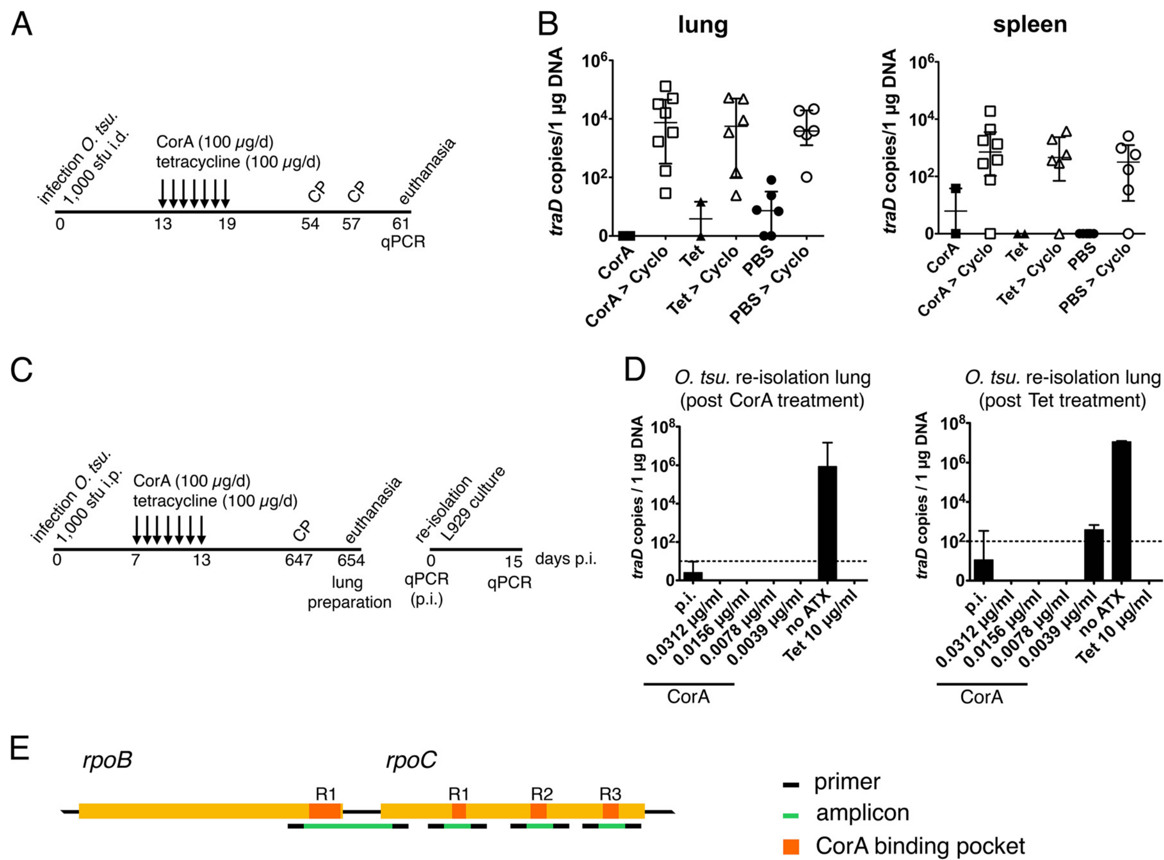

FIG 4 *O. tsutsugamushi* enters latency despite CorA treatment but remains susceptible to CorA. (A) BALB/c mice were infected i.d. with *O. tsutsugamushi* Karp (5,000 SFU) and treated for 7 days with a daily dose of 100 µg CorA, 100 µg tetracycline, or PBS, starting at day 13 p.i. On day 54 and 57 p.i., mice were immunosuppressed by cyclophosphamide (CP) i.p. or left untreated. Mice were euthanized on day 61 p.i. (B) Bacterial loads in lung (left panel) and spleen (right panel) were quantified by *traD* qPCR. Shown are medians ± interquartile range. (C) BALB/c mice were infected i.p. with *O. tsutsugamushi* Karp (5,000 SFU) and treated for 7 days with a daily dose of 100 µg CorA or 100 µg tetracycline. After 647 days p.i., mice were immunosuppressed with cyclophosphamide and euthanized at 654 days p.i. Reisolation of *O. tsutsugamushi* was performed from lung homogenates on irradiated L929 cells in the presence of the indicated concentrations of antimicrobial. (D) Bacterial loads measured by *traD* qPCR in reisolation cultures from one CorA-treated (left) and one tetracycline-treated mouse (right; mean ± SD). No ATX, no antibiotics. (E) Genomic arrangement of *rpoB* and *rpoC* genes and primer strategy. Primers are indicated below.

could not prevent latent infection with *O. tsutsugamushi*. To test whether latent *O. tsutsugamushi* strains acquired antimicrobial resistance subsequent to antibiotic treatment *in vivo*, we immunosuppressed i.p. infected, CorA- or tetracycline-treated mice with cyclophosphamide on day 647 p.i. and reisolated *O. tsutsugamushi* on day 654 p.i. in the presence of CorA, medium, or tetracycline (Fig. 4C). As shown in Fig. 4D, CorA was able to suppress the growth of *O. tsutsugamushi* at concentrations up to its previously determined MIC of 0.0078 µg/ml (Fig. 1B), regardless of whether the mouse had been treated with CorA or was CorA-naive (treated with tetracycline). To corroborate this finding on the molecular level, we sequenced 4 regions within the *rpoB* and *rpoC* genes (Fig. 4E) to which the CorA-binding determinant had been mapped (13), which showed no differences from the wild type (data not shown), further demonstrating that a 7-day treatment with CorA did not select for RNAP mutations. Thus, *O. tsutsugamushi* remained fully susceptible to CorA posttreatment and after entering latency.

CorA-mediated protection against *O. tsutsugamushi* depends on adaptive immunity. We next investigated whether successful antimicrobial treatment against *O. tsutsugamushi* could also be demonstrated in T- and B-cell-deficient SCID mice. SCID and BALB/c mice were infected i.p. with 5,000 SFU of *O. tsutsugamushi* Karp and treated for 7 days with 100 µg CorA, 100 µg tetracycline, or PBS, starting at day 6 p.i. While CorA-treated SCID mice were alive at the end of the treatment phase, all tetracycline-

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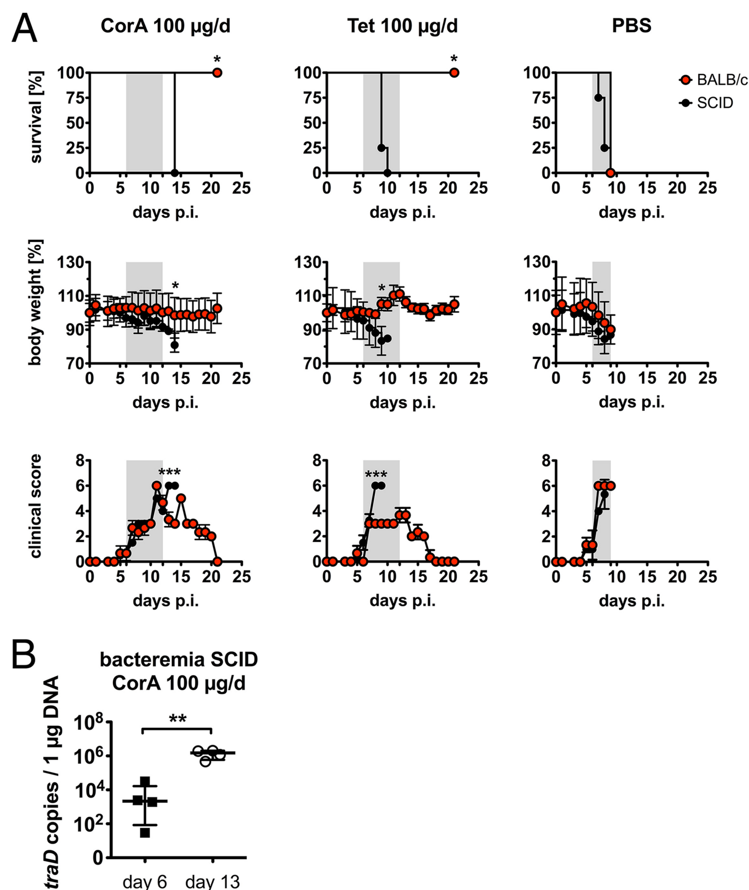

FIG 5 Antimicrobial treatment of *O. tsutsugamushi* infection rescues BALB/c but not SCID mice. SCID mice ($n = 4$) or BALB/c mice ($n = 3$) were infected i.p. with 5,000 SFU *O. tsutsugamushi* Karp and treated for up to 7 days with a daily dose of 100 μg CorA, 100 μg tetracycline, or PBS, starting on day 6 p.i. (A) Shown are survival curves (upper panels), body weight changes (mean ± SD, middle panels), and clinical scores (mean ± SD, lower panels). Survival curves: *, $P < 0.05$ by log-rank (Mantle-Cox) test; weight and score charts: *, $P < 0.05$; ***, $P < 0.001$ by two-way ANOVA and Bonferroni's *post hoc* test. (B) On days 6 and 13 p.i., *O. tsutsugamushi* bacteremia was measured by *traD* qPCR from blood of infected SCID mice that had received 100 μg/day CorA (median ± interquartile range; **, $P < 0.01$ by one-way ANOVA and Bonferroni's *post hoc* test).

treated mice succumbed to the infection by day 10 p.i., before the treatment regimen was completed. The CorA-treated SCID mice died on day 14 p.i. (Fig. 5A, upper panels). Moreover, significantly more weight loss and higher clinical scores, compared to the BALB/c controls, were observed from day 13 p.i. in CorA-treated mice, compared to days 8 and 9 p.i. in tetracycline-treated mice (Fig. 5A, middle and lower panels). In contrast, CorA- or tetracycline-treated BALB/c mice fully recovered from the infection. Also, while CorA inhibited bacterial replication in immunocompetent BALB/c mice (Fig. 3B), SCID mice had >100-fold higher bacteremia on day 13 p.i. compared to day 6 p.i. (Fig. 5B), demonstrating that they were incapable of reducing bacterial replication despite CorA treatment. Thus, survival of acute experimental *O. tsutsugamushi* infection after antimicrobial treatment with CorA (as with tetracycline), as well as sustained inhibition of bacterial replication *in vivo*, relies on the synergistic action of an adaptive immune response.

DISCUSSION

The range of antimicrobial substances for treatment of scrub typhus is limited. Several reports have demonstrated therapeutic failure of doxycycline, the gold standard of therapy in humans, further narrowing the spectrum of choices in certain geographic areas (9, 29). Thus, there is an urgent need for novel compounds that are

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active against *O. tsutsugamushi*. Here, we present a thorough investigation of the myxobacterial α-pyrone compound CorA in its action against *O. tsutsugamushi*, and introduce inhibition of the RNAP switch region as a potent new therapeutic drug target for scrub typhus.

We showed that CorA was effective *in vitro* and *in vivo* against the human-pathogenic *O. tsutsugamushi* strain Karp. The suppression of bacterial growth in the presence of CorA was first demonstrated by confocal microscopy using FISH in infected L929 cells. FISH stains ribosomes, and the absence of a FISH stain indicates absence of significant bacterial metabolic activity. For accurate determination of the MIC, we used the highly sensitive *traD* quantitative real-time PCR (qPCR) for quantification of *O. tsutsugamushi* DNA (23), similar to previously published qPCR-based protocols for susceptibility testing of rickettsiae to antimicrobial agents (30, 31). The MIC of CorA was 0.0078 μg/ml for *O. tsutsugamushi*. We also showed that CorA inhibited the growth of *R. typhi*, but at a 16-fold higher MIC of 0.125 μg/ml, which was comparable to the MIC between 0.1 and 1 μg/ml required for growth inhibition of *Wolbachia* spp. in insect cells (22). Thus, this strikingly low MIC of CorA was specific for *O. tsutsugamushi*, and it is lower than the MICs observed for any other antimicrobial substance that has been so far tested against *O. tsutsugamushi* (32–36). A possible exception may be the tetracycline derivative tigecycline, which was recently shown to inhibit growth *in vitro* of *O. tsutsugamushi* (37), but which awaits susceptibility testing *in vivo*. However, the latter drug is administered intravenously (i.v.) while oral (p.o.) formulations are to be expected for CorA.

At this stage, it remains unclear why *O. tsutsugamushi* is more susceptible than *R. typhi* to CorA. By random mutagenesis of the RNAP subunits RpoB and RpoC in *E. coli*, reduced susceptibility to CorA was related to specific amino acid substitutions in the RNAP switch region (13, 14). Our *in silico* analysis of this binding region (see Fig. S1 in the supplemental material) showed several amino acid variations between *O. tsutsugamushi* and *R. typhi*, and also variations in comparison to *E. coli* and *S. aureus*. Briefly, the RpoC D802E exchange is likely to improve CorA binding in *O. tsutsugamushi*, and the RpoB E1279Q exchange in *R. typhi* may reduce CorA binding in *R. typhi*. In combination, these exchanges could account for the differences in MIC between *O. tsutsugamushi* and *R. typhi* (Fig. S1 and the supplemental material). However, the different susceptibilities toward CorA could also be associated with different levels of permeation of the drug, metabolic differences, or different growth rates of both bacterial species. To investigate whether the RNAP sequence differences between both species really contribute substantially to differences in MIC, MIC analyses with mutated RNAPs will be required.

The human-pathogenic *O. tsutsugamushi* strain Karp produces lethal infections in i.p. inoculated BALB/c mice (23, 26, 38), thus allowing for investigation of the ability of potential therapeutics to protect from fatal outcomes. Titration of CorA in this highly susceptible model showed that a daily dose of 100 μg/mouse (corresponding to 5.48 $\pm$ 0.35 mg/kg) for 7 days initiated at the onset of symptoms was sufficient to protect mice from lethal outcome. When delaying treatment until day 7 p.i., at an advanced stage of infection indicated by a higher clinical score, CorA was able to protect 60% of infected mice. With a sublethal infection dose, 100% of mice were protected by CorA, and body weight loss and clinical scores were significantly improved compared to those of the PBS-treated survivors (Fig. 3A). Moreover, we demonstrated that a short CorA treatment of 2 days protected all infected mice, suggesting that a very short interruption of bacterial replication may be sufficient for therapeutic success. Possibly, a brief inhibition allows priming of adaptive immune mechanisms that efficiently clear the infection. The fact that SCID mice, which lack both T and B cells, were unable to control bacterial growth and therefore succumbed to the infection is consistent with this interpretation. Notably, *O. tsutsugamushi* infections in humans have been observed to respond to single-dose treatments with doxycycline (39), suggesting that short-course treatment with CorA may be equally feasible in humans.

Although *in vitro* MICs of CorA are known for a wide range of pathogens (18),

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effective dose data from *in vivo* models are still scarce. In a previous study, CorA was used at a concentration of 35 mg/kg for depletion of *Wolbachia* endobacteria in filarial nematodes (22). We now show for *O. tsutsugamushi* that CorA is bioavailable and bioactive in a mouse model system at an approximately 7-fold-lower dose. It was also shown here that CorA reduced bacterial loads in ascites and spleen 2 days after treatment, although bacteremia was not significantly lowered. This finding parallels reports in humans where PCR-positive blood samples, representing viable bacteria, are found up to several weeks or even months post treatment with doxycycline or azithromycin, despite clinical recovery (28, 40). Overall, CorA was highly effective at low doses and after only two applications in protecting infected mice from lethal outcome by reducing bacterial replication.

CorA was unable to prevent the establishment of latent *O. tsutsugamushi* infections. Persistence of *O. tsutsugamushi* after antimicrobial treatment has been observed in all commonly used classes of antimicrobials (28, 41). When we induced bacterial reactivation by cyclophosphamide 647 days p.i., viable *O. tsutsugamushi* organisms from lung homogenates could still be inhibited by CorA concentrations down to the previously determined MIC of 0.0078 μg/ml, suggesting that persistence was unrelated to the selection of resistant mutants. As a limitation of our data, we cannot exclude the possibility that resistant mutants were temporarily selected but lost before immunosuppression. However, the probability of such mutation events resulting in a temporarily selected and later lost resistance should be extremely low, and further, the loss of such an acquired mutation would imply that the strains carrying it had a lower fitness compared to that of the nonmutated ones.

By demonstrating the effectiveness of CorA *in vitro* and *in vivo*, our study introduces inhibition of the bacterial RNAP switch region with α-pyrone antibiotics as a new and highly potent therapeutic target for treatment of *O. tsutsugamushi* infections. The rifamycins that also target RNAP, including rifampin, have long been known to be active against *O. tsutsugamushi* and are favored in areas where poor doxycycline responsiveness has been reported (10, 34, 41, 42). Given the crucial role of rifampin as a first-line bacteriostatic drug in tuberculosis treatment, it was recognized early that rifampin is not an ideal standard regimen for treatment of scrub typhus. Tuberculosis is a common infection in areas endemic for scrub typhus, and repeated scrub typhus treatments might carry the risk of inducing rifampin resistance in latently tuberculosis-infected patients (10). Since the binding site of CorA is remote from the rifamycin-binding site (13), the use of an RNAP switch region inhibitor such as CorA for scrub typhus would allow for specific therapy at very low dosage without applying selection pressure in patients that are potentially coinfected with latent tuberculosis.

Altogether, we show here that *O. tsutsugamushi* was highly susceptible to CorA, both *in vitro* and in experimental mouse infections. Similar to tetracycline, CorA required adaptive immunity for antimicrobial action *in vivo* against *O. tsutsugamushi*. CorA did not prevent the establishment of *O. tsutsugamushi* latency, but persistent strains showed unaltered susceptibility to the compound and no mutations of RNAP binding determinants. Our data suggest inhibition of the bacterial RNAP switch region by CorA as a novel and highly potent bacterial target for antimicrobial therapy of scrub typhus.

MATERIALS AND METHODS

Antimicrobial agents. The CorA producer strain *Corallococcus coralloides* B035 was cultivated in a 300-liter bioreactor with 2% Amberlite XAD-16 (DOW Chemicals, Germany) at the Leibniz Institute for Natural Product Research and Infection Biology, Jena. CorA was recovered from the resulting extract by elution with methanol and purified by two-step preparative reverse-phase fast performance liquid chromatography (RP-FPLC) and high-performance liquid chromatography (HPLC) as described before (16). The lyophilized compound was dissolved at a concentration of 10 mg/ml and stored at −80°C in dimethyl sulfoxide before use. Tetracycline (Sigma, Deisenhofen, Germany) was dissolved in PBS at a concentration of 10 mg/ml.

***Rickettsia*/*Orientia* cultures and MIC assays.** *O. tsutsugamushi* Karp and *R. typhi* Wilmington were kindly provided by J. Stenos (Australian Rickettsial Reference Laboratory, Geelong, Australia). Both strains were grown in γ-irradiated murine L929 fibroblasts (obtained from the German Collection of Microor-

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ganisms and Cell Cultures [DSMZ]) in RPMI medium (PAA, Linz, Austria) supplemented with 5% fetal calf serum (FCS; Gibco/Invitrogen, Karlsruhe, Germany), 2% glutamine, and 2% HEPES buffer (both from PAA, Linz, Austria) using 75-cm² flasks and subpassaged every 7 days (*R. typhi*) or 10 days (*O. tsutsugamushi*). For susceptibility assays, γ-irradiated L929 cells were seeded at a concentration of 1×10^5 cells/well in 24-well plates (Greiner Bio-One, Frickenhausen, Germany). Infectious organisms were obtained from continuous cultures after 4 days (*R. typhi*) or 10 days (*O. tsutsugamushi*) of culture. Briefly, infected L929 cells were scraped from two 75-cm² flasks and disrupted by rocking with sterile glass beads. Bacteria were purified by differential centrifugation as described previously (43). Infection was synchronized by centrifugation at $133 \times g$ for 30 min, followed by a replacement of medium (see above) containing 2-fold serial dilutions of CorA (0.0039 to 0.125 μg/ml), 10 μg/ml tetracycline, or unsupplemented medium. An infectious dose was not calculated, but consistency in infectious doses was controlled for by quantification of bacterial loads of infected L929 cells immediately postinfection (p.i.; see following paragraph). The lowest concentration of CorA preventing replication of rickettsiae to copy numbers higher than the p.i. value was interpreted as the MIC.

PCR. To measure the bacterial load in susceptibility assays, samples from infected cell cultures were taken immediately p.i. and after 2, 4, and 6 (*O. tsutsugamushi*) or 3 and 6 (*R. typhi*) days p.i. The cell culture medium was removed, and the cell layer was treated with trypsin (200 μl/well, 5 min at 37°C; PAA, Linz, Austria), followed by thorough pipetting. Cell culture pellets were subjected to DNA extraction following the QiaAmp DNA minikit protocol (Qiagen, Hilden, Germany). The *O. tsutsugamushi* load was quantified by *traD* qPCR (23).

Rickettsiae were quantified using a previously described *ompB*-specific qPCR (44) adapted to the SYBR green format. Briefly, the PCR mix contained, in a total volume of 10 μl, 400 nM each of primer RickS2 (5′-TGTTAAGAATTTCTTTGCTCAAGGT-3′) and RickAS1 (5′-AGTAGCTAAGTCCTGCCATTG-3′; Tibmolbiol, Berlin, Germany), a final concentration of 2 mM $MgCl_2$, 200 μM deoxynucleoside triphosphates (dNTPs), 100 μg/ml bovine serum albumin (BSA), 0.1 of a 1:1,000 dilution of SYBR green I (Invitrogen, Darmstadt, Germany), 0.25 U Hotstar *Taq* DNA polymerase (Qiagen), and 10 ng of template DNA. Enzyme activation at 95°C for 15 min was followed by amplification in 45 cycles of 15 s at 94°C, 15 s at 58°C, and 20 s at 72°C on a LightCycler 480 system (Roche, Mannheim, Germany). Specificity was confirmed by melting curve analysis (0.11°C/s, 5 acquisitions per °C). Results were expressed as *traD* copies/10 ng DNA or transformed as $y = \log_{10}(100 \times \text{copies} + 1)$ and expressed as *traD* copies/μg DNA.

To amplify the CorA binding determinant regions of the β (RpoB) and β′ (RpoC) subunits, the following oligonucleotides were used. *rpoB*: rpoB_for, 5′-TCGACTCCAGTGTTTGATGGG-3′ (nt 3610 to 3630); rpoB_rev2: 5′-CCATGAACGTATCTGCTCAGGA-3′ (nt 87 to 108 [binds in rpoC]); *rpoC* region 1: rpoC_Reg1_for, 5′-GATGCTAGATGCTGGGCGAT-3′ (nt 774 to 793); rpoC_Reg1_rev, 5′-TACCACAACGCTC AAGCTCA-3′ (nt 1170 to 1189); *rpoC* region 2: rpoC_Reg2_for, 5′-TGCTGCTGGAGACCAAACTT-3′ (nt 2118 to 2137); rpoC_Reg2_rev, 5′-GCCGCAACAATACCTACTGC-3′ (nt 2749 to 2768); and *rpoC* region 3: rpoC_Reg3_for, 5′-GCGTCGCTTAATACTGCATC-3′ (nt 3519 to 3539); rpoC_Reg3_rev, 5′-CGGCTGGTACA GGGTTTTA-3′ (4079 to 4097). Products were visualized on precast 2.2% agarose gels (Lonza, Rockland, ME) and bidirectionally sequenced by conventional Sanger sequencing (Seqlab, Göttingen, Germany).

Immunofluorescence and FISH. L929 cells were seeded at a concentration of 0.75×10^5 cells/well on 12-mm glass coverslips (Roth, Karlsruhe, Germany) placed in 24-well plates. Infection with *O. tsutsugamushi* was performed as described above. After 4 days of culture, coverslips were fixed in 4% paraformaldehyde for 30 min at 4°C and stored in PBS. Cells were stained with Alexa-488-conjugated phalloidin (Invitrogen, Darmstadt, Germany) at a dilution of 1:500 in 0.1% Triton X-100/2% FCS-PBS for 90 min at room temperature and fixed with 4% paraformaldehyde for 30 min at 4°C. Fluorescence *in situ* hybridization (FISH) for detection of *Orientia* ribosomal 16S rRNA was performed using the oligonucleotide probe Atto590-5′-TCHACGTCACCGTATYGC-3′ (Biomers, Tübingen, Germany), a modification of a probe designed to recognize the close *Orientia* relative *Sitobion miscanthi* L type symbiont (SMLS) (45). Samples were washed for 5 min in hybridization buffer (0.9 M NaCl, 0.02 M Tris-HCl, 0.01% SDS, and 30% formamide) at 37°C. The probe (50 ng/μl in hybridization buffer) was incubated at 46°C for 90 min in a humid chamber, followed by washing for 15 min in washing buffer (0.102 M NaCl, 0.005 M EDTA, 0.02 M Tris-HCl, and 0.01% SDS) at 46°C. Samples were stained with DAPI (4′,6-diamidino-2-phenylindole; 0.005 mg/ml in 0.01% Triton X-100) for 5 min, washed in distilled water, mounted in ProLong Gold medium (Invitrogen, Darmstadt, Germany) on glass slides, and viewed with an Olympus confocal microscope. Images were analyzed with ImageJ 1.45s software (NIH, Bethesda, USA).

Experimental infections and treatment of mice. For *in vivo* infections, stock preparations of *O. tsutsugamushi* Karp were prepared and quantified by measuring the concentration of spot-forming units (SFU), as described previously (23). All animal experiments were carried out at the BSL3 facility of the Bernhard Nocht Institute for Tropical Medicine (Hamburg, Germany). Female BALB/c mice (age 7 to 9 weeks, starting weight 16 to 20 g) were purchased from Charles River (Sulzfeld, Germany), kept in individually ventilated cages, and infected i.p. with 1×10^3 to 5×10^3 SFU of *O. tsutsugamushi* Karp in a volume of 0.5 ml PBS. Food and water were provided *ad libitum*.

For treatment purposes, CorA and tetracycline were diluted in PBS; mice received a volume of 0.2 ml containing the indicated amount of compound via paramedian i.p. injection at the indicated frequencies.

During the acute disease phase, mice were weighed daily. The clinical score system described by Gharaibeh et al. (43) was used to grade changes of fur (ruffling) and general condition (tiredness, distended abdomen, etc.; for details, see Table S1 in the supplemental material). Mice were sacrificed by cervical dislocation during ketamine-xylazine anesthesia. Organ samples were processed as described previously (23).

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Software and statistics. GraphPad Prism version 5.0 for Mac OS X (GraphPad Software, La Jolla, CA) was used for data analysis. Hypotheses were tested by one-way or two-way analysis of variance (ANOVA) with Bonferroni's *post hoc* correction. Survival curves were analyzed by a log-rank (Mantle-Cox) test. A P value of <0.05 was considered significant. For sequence analysis and ClustalW multiple alignments, MEGA6.06 and BioEdit 7.0.5.3 were used.

Ethics statement. The Animal Protection Commission and the Health Department of the State of Hamburg, Germany approved the animal protocol (approval number 67/12). The protocol adheres to the national regulations (German Animal Welfare Act).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.01732-17.

SUPPLEMENTAL FILE 1, PDF file, 5.8 MB.

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