Macrolide Resistance in *Treponema pallidum* Correlates With 23S rDNA Mutations in Recently Isolated Clinical Strains

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Abstract

Background—High rates of 23S rDNA mutations implicated in macrolide resistance have been identified in *Treponema pallidum* (*T.p.*) samples from syphilis patients in many countries. Nonetheless, some clinicians have been reluctant to abandon azithromycin as a treatment for syphilis, citing the lack of a causal association between these mutations and clinical evidence of drug resistance. While azithromycin resistance has been demonstrated in vivo for the historical Street 14 strain, no recent *T.p.* isolates have been tested. We used the well-established rabbit model of syphilis to determine the in vivo efficacy of azithromycin against 23S rDNA mutant strains collected in 2004–5 from patients with syphilis in Seattle, WA.

Methods—Groups of 9 rabbits were each infected with a strain containing 23S rDNA mutation A2058G (strains UW074B, UW189B, UW391B) or A2059G (strains UW228B, UW254B, and UW330B), or with one wild type strain (Chicago, Bal 3, and Mexico A). After documentation of infection, 3 animals per strain were treated with azithromycin; 3 were treated with benzathine penicillin G; and 3 served as untreated control groups. Treatment efficacy was documented by darkfield microscopic evidence of *T. pallidum*, serological response, and rabbit infectivity test.

Results—Azithromycin uniformly failed to cure rabbits infected with strains harboring either 23S rDNA mutation, although benzathine penicillin G was effective. Infections caused by wild type strains were successfully treated by either azithromycin or benzathine penicillin G.

Conclusions—A macrolide resistant phenotype was demonstrated for all strains harboring a 23S rDNA mutation, demonstrating that either A2058G or A2059G mutation confers in vivo drug resistance.

Keywords

syphilis; drug resistance; treatment

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INTRODUCTION

Benzathine penicillin G is an inexpensive and effective treatment for syphilis, and there is no confirmed clinical or laboratory evidence of penicillin resistance in *Treponema pallidum (T.p.)*. This treatment, however, requires painful intramuscular injections, and clinicians have long sought a single dose oral regimen for syphilis. Azithromycin, a long-acting oral ketolide antibiotic, is effective against the standard laboratory strain of *T.p.* (Nichols) in the rabbit model[1] and several early studies confirmed the efficacy of azithromycin for syphilis treatment in non-pregnant adults.[2–4]

When syphilis rates in the US began to increase in 2000, single dose azithromycin began to be used more widely to treat syphilis. This regimen was particularly attractive for partner-delivered treatment of sexual contacts. In 2002, however, azithromycin treatment failures were identified in San Francisco, and samples from these individuals contained the 23S A2058G mutation.[5] Samples from San Francisco, Dublin, and Seattle documented a high prevalence of the A2058G mutation in circulating strains of *T.p.*, with an increasing frequency of the mutation over time.[6–9] Harboring a strain with this mutation was associated with past macrolide exposure.[10] A second 23S rDNA mutation, A2059G, was identified in a secondary syphilis patient who failed therapy with the macrolide antibiotic spiramycin, and the mutation was subsequently identified in 18% of samples from patients with syphilis in the Czech Republic.[11]

The development of a rapid, isolation-independent molecular method to test patient samples led to widespread reports of varying prevalence rates of these mutations. For example, up to 97% of samples tested in Shanghai, China, have the A2058G mutation,[12] whereas this mutation has been identified in very low frequency in Taiwan.[13,14] The A2059G mutation has been seen in multiple sites, although in lower frequency.[9,15] The rise in syphilis rates, particularly among men who have sex with men (MSM), has resulted in a corresponding observation of 23S rDNA mutations in patient samples in London,[16] Dublin,[8] South Africa,[17] US,[18] Seattle,[9] and China.[19]

Despite the reported association of the A2058G and A2059G 23S rDNA mutations with treatment failure after treatment with macrolides,[20] there has been controversy about the clinical relevance of the mutations.[21] In a paper describing a large multi-site equivalence study of azithromycin vs. benzathine penicillin G for treatment of early syphilis, the authors suggest that the question of whether 23S mutations contribute to syphilis treatment failure among individuals treated with azithromycin remains unresolved.[21]

The rabbit model of syphilis offers a definitive means to examine the correlation between 23S rDNA mutations and efficacy of azithromycin therapy. Our ongoing syphilis research programs have included isolation of *T. pallidum* strains from patient samples (blood, cerebrospinal fluid, chancre exudate) for many years, and these strains have been tested for 23S rDNA mutations.[9,10] We used six of these strains, containing either the A2058G or A2059G mutation, to examine in vivo susceptibility of *T. pallidum* to azithromycin in the rabbit model.

Materials and Methods

Strains

Six *T.p.* strains isolated from blood of patients with untreated syphilis were selected to represent the A2058G and A2059G 23S mutations and, when possible, to represent different molecular strain types within groups (Table 1).[9,10] Wild type, historical strains were obtained originally from Paul Hardy and Ellen Nell at Johns Hopkins University. These strains had not been tested previously for susceptibility in vivo to azithromycin. All strains were stored as glycerol stocks in liquid nitrogen prior to propagation in rabbits as previously described.[22]

Molecular demonstration of 23S rDNA mutation

23S rDNA mutations were confirmed using the published restriction fragment length polymorphism (RFLP) methods previously described.[6,11] Briefly, an initial 23S rDNA polymerase chain reaction (PCR) product specific to *T.p.* genomic DNA served as template for a nested PCR. For restriction enzyme digestion, the A2058G mutation was detected with MboII (New England Biolabs, Ipswich, MA) and the A2059G mutation with BsaI (NEB). Wild type amplicons were not cut by either enzyme.

Infections

Outbred adult male New Zealand white rabbits (2.5 – 3.5 kg) were obtained from R & R Rabbitry, Stanwood, WA. Initially, each rabbit had negative serological results by fluorescent treponemal antibody-absorption (FTA-ABS) and Venereal Disease Research Laboratory (VDRL, Becton, Dickinson and Company, Sparks, MD) tests. Rabbits were fed antibiotic-free food and water and housed at 16 – 18°C. These studies were approved by the University of Washington Institutional Animal Care & Use Committee, and care was provided in full accordance with the Guide for the Care and Use of Laboratory Animals.

Groups of 9 rabbits were infected intradermally (ID) with one of the three wild type strains or with one of two Seattle isolates that contained A2059G mutations. Intradermal infection is preferable to intratesticular infection because the skin lesions can be readily aspirated for darkfield microscopic examination for the presence of viable *T.p.* to determine how quickly the antibiotic treatment results in bacterial clearance (see Table 2). However, ID infection with the other four Seattle strains (3 containing the A2058G mutation and one strain with the A2059 mutation) does not reproducibly induce robust skin lesions; therefore, groups of 9 rabbits were infected intratesticularly with each of these strains.

Intradermal Infections (ID)—Wild Type (Chicago, Bal 3, Mexico A); A2059G mutation (UW228B, UW330B): Rabbit backs were clipped free of fur, and each rabbit was infected ID at 10 sites with a 0.1mL volume of 10^7 *T.p.*/mL (10^6 *T.p.* per site). Lesions were examined daily for size and progression for 10 days after initiation of treatment. Once lesions were evident, aspirates were taken for darkfield (DF) microscopic examination prior to treatment and every 2 days after initiation of treatment; all rabbits in the treatment studies had treponemes detectable by DF prior to initiation of treatment. After treatment, rabbits whose lesion aspirates became and remained DF-negative on three consecutive time points

were declared DF-negative and were no longer examined by this method. The microscopist was blind to the treatment status and infecting strain of the animals from which the samples were collected.

Intratesticular Infections (IT)—A2058G mutation (UW074, UW189, UW391); A2059G mutation (UW254B): To match the intradermal infection experiments, the same total number of bacteria (10^7) was delivered into one testicle per rabbit. Clinical evidence of orchitis (monitored twice weekly) or darkfield evidence of *T.p.* in a testicular aspirate was considered to be proof of active infection prior to initiation of treatment.

Treatment

For each strain tested, there were three treatment groups with 3 rabbits per group: control (no treatment), azithromycin, benzathine penicillin G. The timing of initiation of treatment was dependent upon clinical evidence of infection (DF+ aspirate or orchitis), thus the timing of treatment initiation varied among strains. Azithromycin-treated animals received one 45mg capsule PO daily (15 mg/kg), roughly equivalent (w/w) to 1 g/day in humans, for 10 days and consistent with our previous study.[1] Benzathine penicillin G (BPG)-treated animals received a single IM injection (200,000 units; equivalent w/w to 4.8 million units for humans).[1]

Serological testing

Blood was collected from all rabbits every two weeks post infection for 8–14 weeks for quantitative VDRL testing. Serological assays for all time points were performed on the same day to minimize test-to-test variability; the technologist was blinded to the treatment status of the animals from which the samples were collected. Mean $\log_2$ titers +/− SE were calculated for each treatment group at each time point: nonreactive= −1.0; weakly reactive= −0.5; (reactive at a titer of 1:1) R1=0 and R2=1, etc.

Rabbit Infectivity Test

Upon completion of serological monitoring, all rabbits were euthanized for rabbit infectivity testing (RIT) as described previously.[23] Popliteal lymph nodes from each rabbit were minced in normal rabbit serum, pooled by group, and each pool was injected IT into a naïve rabbit for detection of persistent infection in the donors. Development of either DF+ orchitis or VDRL and FTA-ABS seroconversion in the recipient rabbit during a 3 month observation period was considered to be evidence of transferred infection. Observation was discontinued in a recipient rabbit when either of these criteria was met. When recipient rabbits failed to develop evidence of *T.p.* infection by the end of the observation period, the donors were assumed to be cured by antibiotic treatment.

RESULTS

Three criteria were used to determine susceptibility of a *T.p.* strain to antibiotic treatment: disappearance of *T.p.* by DF microscopic examination of lesion aspirates; four-fold reduction in VDRL titer or reversion to nonreactive following treatment; and failure of tissues to transfer active infection to recipient rabbits. Table 2 summarizes the darkfield

microscopic evidence of treponemal infection following completion of treatment in rabbits infected intradermally. All rabbits treated with BPG had no detectable *T.p.* in lesion aspirates by day 2 after treatment initiation and remained DF-negative for two subsequent examinations. After treatment with azithromycin, rabbits infected with wild type strains had no detectable *T.p.* in lesion aspirates by day 4 after initiation of treatment and remained DF-negative for two subsequent examinations. In contrast, all rabbits infected ID with the two strains containing the A2059G mutation and treated with azithromycin had detectable *T.p.* in lesion aspirates throughout the 10-day observation period. All untreated intradermally-infected rabbits had detectable *T.p.* in lesion aspirates throughout the 10-day observation period.

In untreated individuals with syphilis, serum VDRL or rapid plasma reagin (RPR) titers increase through the secondary stage of disease. After treatment, a 4-fold reduction in titer is an indication of the efficacy of treatment. A similar picture is seen in the rabbit model of syphilis, regardless of the route of infection. Figure 1 shows the mean+/− SE $\log_2$ titers of serum VDRL antibody over the course of infection and treatment for a representative strain in each 23S rDNA mutation group. Serological evidence of treatment success was seen following BPG treatment for all strains, and following azithromycin treatment of wild type strains. Rabbits infected with strains containing either 23S rDNA mutation failed to show a significant reduction in titer post azithromycin, following the same pattern of increasing titer as seen in untreated animals infected with that same strain.

The gold standard test for persistent *T.p.* is the highly sensitive RIT.[23] As shown in Table 3, tissue from BPG treatment groups for all strains failed to transfer infection to recipient rabbits during the 3-month observation period, indicating effective treatment by BPG for all strains. Conversely, all rabbits receiving tissues from untreated animals for all strains developed either DF+ orchitis or seroconversion (VDRL and FTA-ABS). Azithromycin treatment of rabbits infected with any of the 3 wild type strains was effective (RIT-negative), but tissues from animals infected with any of the 6 strains containing A2058G or A2059G 23S rDNA mutations remained infectious for the recipient animals, indicating functional resistance to azithromycin in treated animals.

DISCUSSION

We undertook this study to address uncertainty in the scientific and medical communities regarding the clinical relevance of 23S rDNA mutations in *T. pallidum*. In other bacteria, the A2058G and A2059G 23S rDNA mutations confer functional resistance to macrolide antibiotics, yet concern has been raised in the syphilis literature about whether the presence of these mutations in *T.p.* conveys a macrolide resistant phenotype.[21]

The Street 14 strain, isolated in 1977, is resistant to macrolide antibiotics in vivo, but demonstration of resistance in more recently isolated strains lends particular relevance to the management of today's patients. In many areas, the prevalence of strains containing a 23S rDNA resistance mutation is greater than 80–85%.[9,12,24,25] Surveillance for these mutations can provide practical information as illustrated by a recent report from Taiwan, where azithromycin was able to be used for syphilis treatment during a recent BPG shortage

because of a known low frequency of strains carrying mutations.[26] In the US and Western Europe, the strains containing 23S rDNA mutations are reported in the MSM population consistent with the epidemiology of syphilis in these regions, although studies of separate demographic groups have not been reported. The persistence of the mutations in the population over many years suggests that the A2058G and A2059G mutations in *T. p.* may not confer a significant fitness disadvantage. If true, then these resistant strains may continue to circulate widely in the future, even in the absence of antibiotic pressure.

Our results clearly link in vivo azithromycin resistance to 23S rDNA mutations in 6 recently isolated strains. To avoid the possibility that we were testing separate isolates of the same circulating strain, we endeavored to select strains with different molecular types when possible. Although three strains with A2058G mutations and different molecular type were available, all of our isolates containing A2059G were of the same type. They were isolated over the course of 12-month period, so they may or may not represent a single strain. Nonetheless, all three isolates were clearly resistant to azithromycin. To ensure rigor in our investigations, we used multiple methods for demonstrating persistence of infection after treatment: darkfield microscopic examination of material aspirated from skin lesions or infected testes, the course of serum VDRL titers before and following treatment, and the RIT, which is the gold standard measure of the presence of infectious *T.p.* in a treated person or experimental animal.

Penicillin remains the preferred treatment for syphilis. Benzathine penicillin G is the form of penicillin that provides treponemicidal levels of penicillin in blood for multiple weeks following a single administration. This long duration of drug levels is considered necessary because *T.p.* divides very slowly (30–33 hours),[27] and the drug must be present during cell wall synthesis to be effective. A recent study by Tipple *et al.* showed that, following BPG treatment of three primary and secondary syphilis patients, blood was clear of *T.p.* DNA by PCR after a mean of 32 hours, and chancre exudate from the single patient with primary syphilis was cleared of DNA by 56 hours.[28] Consistent with the data in humans, rabbits treated with BPG have no *T.p.* detectable in lesion exudate by DF microscopy ~1 day post-BPG.[6] The PCR assay used in the Tipple study could be more sensitive than darkfield microscopy or may have detected residual nucleic acids from dead organisms. Strains with proven penicillin resistance have not been isolated from patients, and clinical failures are uncommon following BPG treatment.

Despite the efficacy of penicillin, the availability of an effective, oral single dose treatment for syphilis would be welcomed by patients and clinicians. It was hoped that azithromycin would be that drug, but the high frequency of 23S rDNA mutations, particularly in light of our findings, makes azithromycin inappropriate in most geographical regions. The rapid dissemination of *T.p.* strains containing these mutations over many countries and multiple continents has essentially removed azithromycin and other macrolides from the repertoire of drugs that can be used to treat syphilis. Continued surveillance for 23S mutations is important for tracking the movement of resistant strains and for identifying the development of new mutations.

Acknowledgments

Abbreviations

BPG	benzathine penicillin G
DF	darkfield microscopy
FTA-ABS	fluorescent treponemal antibody-absorption
PO	per os: by mouth
RIT	rabbit infectivity test
T.p.	Treponema pallidum
VDRL	Venereal Disease Research Laboratory

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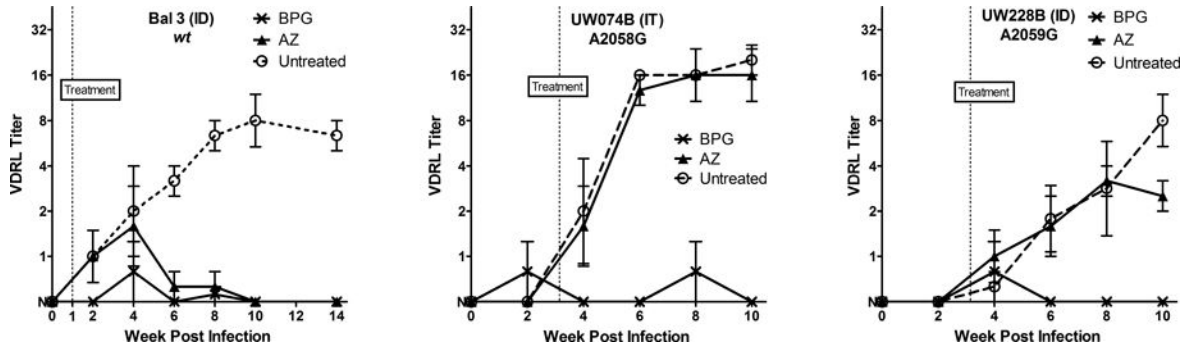29. Turner, TB.; Hollander, DH. Biology of the Treponematoses. Geneva: World Health Organization; 1957.

FIGURE 1.

Mean (+/− SE) serum VDRL titers in untreated- (**open circle**), BPG- (**x**), and azithromycin-(**triangle**) treated rabbits. The data represent 3 rabbits per treatment group, and mean serum VDRL titers are shown through 10–14 weeks post-infection, and at least six weeks following initiation of treatment. One representative strain is shown for each 23S group: wild type, A2058G, and A2059G, as noted. For calculating mean +/− SE, titers were converted to $\log_2$ with nonreactive= −1.0; weakly reactive= −0.5; R1= 0, and R2=1, etc. Antilogs of mean +/−SE of titers are shown in this figure with nonreactive set at y=0.

TABLE 1

Strains used in this study

Group	Strain	Molecular Strain Type	Source[*]
Wild Type	Bal 3	12a/c	Baltimore, unknown
	Chicago[29]	14a/a	Chicago, 1951
	Mexico A[29]	14d/e	Mexico, 1953
A2058G	UW074B	14d/g	Seattle, 2004
	UW189B	13d/d	Seattle, 2004
	UW391B	14d/f	Seattle, 2005
A2059G	UW228B	15d/f	Seattle, 2004
	UW254B	15d/f	Seattle, 2004
	UW330B	15d/f	Seattle, 2005

[*]Location, year

TABLE 2

Darkfield detection of *T.p.* in intradermally infected rabbits following treatment

Strain	Benzathine Penicillin G	Azithromycin	Untreated
Wild Type			
Chicago	0/3 *	0/3	3/3
Mexico A	0/3	0/3	3/3
Bal 3	0/3	0/3	3/3
A2059G			
UW228B	0/3	3/3	3/3
UW330B	0/3	3/3	3/3

*Number of rabbits with DF-detectable *T.p.* in lesion aspirates by day10/number of rabbits per group.

TABLE 3

Summary of Results of Rabbit Infectivity Test

Strain	Benzathine penicillin G	Azithromycin	Untreated
Wild Type			
Chicago	N*	N	DF+ (1mo)
Mexico A	N	N	R16 (2mo)
Bal 3	N	N	R8 (2mo)
A2058G			
UW189B	N	R8 (2mo)	R16 (2mo)
UW391B	N	R8 (2mo)	R8 (1mo)
UW074B	N	R16 (3mo)	R16 (1mo)
A2059G			
UW228B	N	R2 (1mo)	R4 (1mo)
UW254B	N**	R16 (7wk)	R64 (7wk)
UW330B	N	R16 (1mo)	DF+ (1 mo)

*
N = Nonreactive serum VDRL at three months post-transfer, R = Reactive serum VDRL with reciprocal titer (time post-transfer at which seroconversion was first detected), DF+ = Darkfield positive aspirate from testicular aspirate; no titer available.

**
Euthanized at 7wk due to unrelated illness.