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Susceptibilities of *Mycoplasma hominis*, *Mycoplasma pneumoniae*, and *Ureaplasma urealyticum* to New Glycylcyclines in Comparison with Those to Older Tetracyclines

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The glycylcyclines are new tetracycline derivatives that include the *N,N*-dimethylglycylamido derivative of minocycline (DMG-MINO) and the *N,N*-dimethylglycylamido derivative of 6-demethyl-6-deoxytetracycline (DMG-DMDOT). The susceptibilities of *Mycoplasma pneumoniae*, *Mycoplasma hominis*, and *Ureaplasma urealyticum* to DMG-MINO, DMG-DMDOT, tetracycline, doxycycline, and minocycline were determined by the agar dilution method. The glycylcyclines with MICs at which 50% of the isolates are inhibited of 0.25 to 0.5 μg/ml for *M. pneumoniae* were two- to fourfold more active than tetracycline and had the same activity as minocycline and doxycycline. Tetracycline-susceptible *M. hominis* strains were four- to eightfold more susceptible to the glycylcyclines (0.12 to 0.25 μg/ml) than to tetracycline. Strains of *M. hominis* known to be resistant to tetracycline, doxycycline, and minocycline because of the *tet*(M) determinant were as susceptible to the glycylcyclines as the tetracycline-susceptible strains. For tetracycline-susceptible *U. urealyticum* strains, the glycylcyclines showed the same activity as tetracycline (MICs at which 50% of the isolates are inhibited of 1 to 2 μg/ml). Tetracycline-resistant strains of *U. urealyticum* were resistant to doxycycline and minocycline and showed variable susceptibility to the glycylcyclines (range, 0.5 to 32 μg/ml). In view of the increasing resistance of *M. hominis* and *U. urealyticum* strains to tetracyclines, the glycylcyclines have promise, pending assessment of their pharmacokinetic and safety profiles.

Because mycoplasmas lack a cell wall, the spectrum of antibiotics that can be used for treating mycoplasmal infections is limited. The tetracyclines have been one of the few antibiotic groups considered to be generally active against the human and animal mycoplasmas. However, high-level tetracycline resistance is increasing in clinical isolates of *Mycoplasma hominis* (7, 18) and *Ureaplasma urealyticum* (23, 26). Resistance in both cases is due to the acquisition of *tet*(M) (23, 25), which protects the ribosome from tetracycline (28). The problem of tetracycline resistance is broad because many bacterial pathogens (e.g., gonococci, streptococci, enterococci, and staphylococci) that infect the same respiratory and genital sites as mycoplasmas show high-level resistance to tetracyclines through a variety of transposon- and plasmid-mediated mechanisms (21, 28). In devising alternative antimicrobial therapies for resistant bacterial pathogens, the susceptibilities of mycoplasmas must be considered because the clinical picture may not permit a clear distinction between mycoplasmal and bacterial infections.

The glycylcyclines are new derivatives of tetracycline that are highly active on certain bacteria known to be tetracycline resistant by either efflux or ribosomal protection mechanisms (29). Two glycylcyclines have been described (29): the *N,N*-dimethylglycylamido derivative of minocycline (DMG-MINO) and the *N,N*-dimethylglycylamido derivative of 6-demethyl-6-deoxytetracycline (DMG-DMDOT). We determined the susceptibilities of *Mycoplasma pneumoniae*, *M. hominis*, and *U. urealyticum* to DMG-DMDOT and DMG-MINO compared with their susceptibilities to tetracycline, minocycline, and doxycycline by using both tetracycline-susceptible and tetracycline-resistant strains.

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MATERIALS AND METHODS

Mycoplasmas and media. The strains of *M. pneumoniae*, *M. hominis*, and *U. urealyticum* used in this study were clinical isolates and prototypic strains as described previously (12, 17). *Mycoplasma arginini* G-230 (3) was obtained from M. F. Barile. The *tet*(M)-containing strain of *M. hominis*, GX 55, was produced in this laboratory by conjugation with a *tet*(M)-containing *Enterococcus faecalis* strain (24). The mycoplasmal media contained 20% horse serum and 10% fresh yeast dialysate in a soy peptone base (11). H broth (11) at pH 7.5 to 7.6 contained 5 mM glucose when used for *M. pneumoniae* and 5 mM arginine when used for *M. hominis* and *M. arginini*. For *U. urealyticum*, U broth (11) contained 10 mM MES (2[*N*-morpholino]ethanesulfonic acid) buffer and 5 mM urea at pH 6.3 to 6.4. The agar media were H agar at pH 7.5 to 7.6 for *M. hominis* and *M. pneumoniae* and U agar at pH 6.3 for *U. urealyticum* (11). The pH of the medium was measured with a surface electrode after incubation under the conditions described above for 24 h.

Agar dilution susceptibility testing. MICs were determined by the agar dilution method as adapted for mycoplasmas (12, 17) by using a Steers' replicator with serial 10-fold dilutions of organisms. The inocula used were from actively growing cultures. The MIC was the amount of antibiotic that completely inhibited the formation of colonies on plates known to have been inoculated with 30 to 300 CFU in 4 days for *U. urealyticum*, 5 days for *M. hominis*, and 14 days for *M.*

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TABLE 1. Susceptibilities of *M. pneumoniae*, *M. hominis*, and *U. urealyticum* to the glycylcyclines and tetracyclines

Organism	Compound	No. of strains	MIC (μg/ml)[a]		
			Range	50%	90%
M. pneumoniae	DMG-MINO	53	0.25–1.0	0.5	1.0
	DMG-DMDOT	53	0.125–2.0	0.25	0.5
	Tetracycline	53	0.5–2.0	1.0	2.0
	Doxycycline	20	0.25–0.5	0.5	0.5
	Minocycline	53	0.25–2.0	0.5	1.0
M. hominis (tetracycline susceptible)	DMG-MINO	30	0.12–0.25	0.25	0.25
	DMG-DMDOT	30	0.12–0.25	0.12	0.25
	Tetracycline	30	0.12–2.0	1.0	2.0
	Doxycycline	25	0.12–0.5	0.12	0.5
	Minocycline	30	0.06–0.12	0.12	0.12
U. urealyticum (tetracycline susceptible)	DMG-MINO	42	0.5–2.0	1.0	2.0
	DMG-DMDOT	42	0.25–4.0	2.0	2.0
	Tetracycline	42	0.25–4.0	1.0	4.0
	Doxycycline	24	0.25–1.0	0.5	1.0
	Minocycline	42	0.06–1.0	0.25	0.5

[a] 50% and 90%, MIC_{50} and MIC_{90}, respectively.

pneumoniae. Agar plates were incubated in air, except for experiments requiring pH 7.1 to 7.2 in which H agar plates were incubated in an atmosphere of 2.5% CO_2 in air. The results shown in the tables are taken from experiments in which all of the antibiotics had been simultaneously tested.

Antibiotics. Two glycylcyclines (29), CL 329998 (DMG-MINO) and CL 331002 (DMG-DMDOT), and minocycline were obtained from Lederle Laboratories, American Cyanamid Co., Pearl River, N.Y. Doxycycline and tetracycline were purchased from Sigma Chemical Co., St. Louis, Mo. Antibiotics were prepared freshly for each experiment and were dissolved in water.

RESULTS

Susceptibilities of mycoplasmas. *M. pneumoniae* strains were two- to fourfold more susceptible to the glycylcyclines, doxycycline, and minocycline (MICs at which 50% of the isolates were inhibited [MIC_{50}s] of 0.25 to 0.5 μg/ml [Table 1]) than to tetracycline. The distribution of susceptibilities of isolates of *M. hominis* to tetracycline was strongly bimodal, as noted before (18), with MICs for susceptible strains ranging from 0.12 to 2.0 μg/ml (MIC_{50} of 1 μg/ml) and with resistant strains showing susceptibilities of 32 to >64 μg/ml (compare Tables 1 and 2). Tetracycline-susceptible *M. hominis* strains were four- to eightfold more susceptible to the glycylcyclines, minocycline, and doxycycline (MIC_{50}s of 0.12 to 0.25 μg/ml) than they were to tetracycline (Table 1).

TABLE 2. Susceptibility of tetracycline-resistant strains of *M. hominis* and *U. urealyticum* to glycylcyclines

Compound	*M. hominis*[a]		*U. urealyticum*[b]	
	MIC_{50} (μg/ml)	Range	MIC_{50} (μg/ml)	Range
DMG-MINO	0.25	0.12–0.25	8	1–32
DMG-DMDOT	0.25	0.12–0.25	4	0.5–32
Tetracycline	64	64–>64	>64	32–>64
Doxycycline	32	4–32	64	16–64
Minocycline	>64	64–>64	32	16–>64

[a] Ten strains were tested, except for doxycycline for which 6 strains were used.
[b] Eight strains were tested, except for doxycycline for which five strains were used.

Tetracycline-resistant strains of *M. hominis* and *M. arginini*. Tetracycline-resistant isolates of *M. hominis* were resistant to doxycycline and minocycline (Table 2) but were as susceptible to the glycylcyclines as the tetracycline-susceptible strains (Table 1). A tetracycline-susceptible strain of *M. hominis* was made tetracycline resistant by conjugation with a *tet*(M)-containing strain of *Streptococcus faecalis* (24). The *tet*(M) determinant was proven to be transferred to *M. hominis* (24), and the transconjugant was resistant to tetracycline, doxycycline, and minocycline, but it was as susceptible to the glycylcyclines as the tetracycline-susceptible parent strain (Table 3). A series of isogenic step mutants of *M. arginini* (an organism of goats and sheep that is closely related to *M. hominis*) were available. These mutants were isolated by plating mycoplasmas on agar medium containing tetracycline at increasing concentrations of one to four times the MIC for the parent strain. Sequential selection of mutants on higher concentrations of tetracycline yielded three step mutants (Table 3), for each of which tetracycline MICs were two- to fourfold greater than those for their individual parent. Overall, the mutants produced were 2 to >16 times more resistant to tetracycline than the parent strain. They were proven not to contain *tet*(M) (25). As the mutants increased in resistance to tetracycline, they also showed increases in resistance to minocycline, DMG-MINO, and DMG-DMDOT. However, each of the step mutants was 8- to 32-fold more susceptible to minocycline and the glycylcyclines than to tetracycline.

***U. urealyticum*.** Susceptible strains of *U. urealyticum* were as susceptible to the glycylcyclines as to tetracycline (MIC_{50}s of 1 to 2 μg/ml) and were two- to fourfold more susceptible to doxycycline and minocycline (Table 1). Tetracycline-resistant strains (Table 2) were resistant to doxycycline and minocycline (MIC_{50}s of 32 to 64 μg/ml) and were susceptible to DMG-MINO at 8 μg/ml and to DMG-DMDOT at 4 μg/ml, values two- to fourfold greater than those for susceptible strains (compare Tables 1 and 2). The susceptibilities of the tetracycline-resistant ureaplasmas to the glycylcyclines showed a much broader range (0.5 to 32 μg/ml) than that observed for susceptible ureaplasmas (0.25 to 4 μg/ml).

TABLE 3. Susceptibility of isogenic strains of *M. arginini* and *M. hominis* to tetracyclines and glycylcyclines

Strain	Susceptibility (μg/ml) to:				
	DMG-MINO	DMG-DMDOT	Tetracycline	Minocycline	Doxycycline
M. arginini G-230					
Parent	0.25	0.25	4	0.5	ND[a]
Step 1[b]	0.5	0.5	8	0.5	ND
Step 2	0.5	0.5	16	2.0	ND
Step 3	4.0	4.0	>64	8.0	ND
M. hominis GX 55					
Parent	0.12	0.12	0.12	0.12	0.5
tet(M) transconjugant[c]	0.12	0.12	>64	>64	32

[a] ND, not done.
[b] Resistant mutants were selected by plating undiluted actively growing cultures of *M. arginini* G-230 on agar medium containing tetracycline at one to four times the MIC for the parent strain. Resistant colonies were rare and were detected as late as 14 days.
[c] The transconjugant resulted from a mating of *M. hominis* GX 55 with a *tet*(M)-carrying strain of *E. faecalis* (24).

Effect of pH on susceptibility of *M. hominis*. Mycoplasmas are customarily grown on agar medium with a pH of 7.6 or greater. Ureaplasmas grow best at pH 6.3 to 6.4 and will not form visible colonies on medium with a pH of 7.0 or greater, indicating a potential problem for comparison of results between mycoplasmas and ureaplasmas if pH has an effect on activity. We determined the effect of pH by using *M. hominis*, which grows readily on agar at pH 6.0 through 8.0 (Table 4). Small effects were repeatedly seen: *M. hominis* was more susceptible to tetracycline (twofold) and doxycycline (fourfold) at pH 6.2 to 6.3 than at pH 7.5 to 7.6. In contrast, *M. hominis* was more susceptible to DMG-MINO (twofold) and DMG-DMDOT (fourfold) at pH 7.5 to 7.6 than at pH 6.2 to 6.3.

Stability of tetracyclines and glycylcyclines. Because mycoplasmas grow slowly, taking from 2 to 14 days to form colonies, inactivation of the antimicrobial agent might occur during incubation. We investigated this possibility by holding agar plates for as long as 4 days at either room temperature or 37°C before inoculating the plates. No significant differences in MICs for *M. hominis* were seen (not shown) at either pH 6.1 or pH 7.5.

DISCUSSION

The major finding of this study was that the glycylcyclines were highly active against naturally occurring, highly tetracycline-resistant *M. hominis* strains that contained the resistance determinant *tet*(M). This was confirmed when we found that a *tet*(M) transconjugant of *M. hominis* was as susceptible as the parent strain to the glycylcyclines (MIC of 0.12 μg/ml) but was totally resistant to tetracycline, doxycycline, and minocycline (MICs of 32 to >64 μg/ml [Table 3]). Similar results have been observed for isogenic strains of *Escherichia coli* and *Staphylococcus aureus*, in which the *tet*(M) transconjugants were as susceptible to glycylcyclines as the parent strains (29). The glycylcyclines have been shown to be effective against bacteria containing other tetracycline resistance determinants (29). Thus far, *tet*(M) has been the principal factor in high-level tetracycline resistance for *M. hominis* and *U. urealyticum* (6, 23, 25, 26). The glycylcyclines were as active against tetracycline-susceptible *M. hominis* strains as doxycycline and minocycline (0.06 to 0.25 μg/ml [Table 1]) and were four- to eightfold more active than tetracycline (1 to 2 μg/ml). In contrast to the glycylcyclines, minocycline and doxycycline were 16- to 32-fold less active against tetracycline-resistant clinical isolates of *M. hominis* than against susceptible strains (compare Tables 1 and 2). Some *tet*(M)-containing *M. hominis* strains have been classified as doxycycline susceptible but tetracycline resistant because the MICs for the strains fall within the high end of the susceptibility range of bacteria for doxycycline (6). In our study, *M. hominis* isolates with *tet*(M) showed greatly increased resistance (8- to ≥32-fold) to doxycycline but the MICs for the strains were lower than those for tetracycline, an effect possibly explained by the greater activity of doxycycline and minocycline against susceptible strains (Table 1). In addition, the transconjugant *M. hominis* strain was ≥32-fold more resistant to doxycycline and minocycline than the parent strain.

Tetracycline-susceptible ureaplasmas appeared to be susceptible to the glycylcyclines and tetracycline at a MIC_{50} of 1 μg/ml; however, the MIC_{90} for the strains was a more marginal 2 to 4 μg/ml (Table 1). They were more susceptible to doxycycline and minocycline at MIC_{50}s of 0.25 to 0.5 μg/ml. Since ureaplasmas were tested at pH 6.3 and *M. hominis* was tested at pH 7.6, the pH of the medium was a possible factor. When strains of *M. hominis* were tested at various pHs, the glycylcyclines were two- to fourfold less active at pH 6.2 to 6.3 than at pH 7.5 to 7.6, whereas doxycycline and tetracycline were two- to fourfold more active at acid pH (Table 4). The pH effect for the glycylcyclines is the reverse of that for tetracycline, which is known to be more active against bacteria at acidic pH (1). The differing pH effects could account for the relatively low susceptibilities of ureaplasmas to glycylcyclines. These results suggest that it is important to consider pH in susceptibility testing of mycoplasmas and ureaplasmas. Ureaplasmas appeared to be far less susceptible to erythromycin when tested at pH 6.3 than at pH 7.0 (16). Tetracycline-resistant ureaplasmas were resistant to minocycline and doxycycline, and they showed a broad range of susceptibilities to the glycylcyclines, with some strains appearing susceptible and others resistant (Table 2). The most resistant of the strains was serovar 9, which has had the most laboratory transfers (26).

TABLE 4. Effect of pH on susceptibility of 17 strains of *M. hominis* to glycylcyclines and tetracyclines

Antimicrobial agent	MIC_{50} (μg/ml) at pH:		
	6.2–6.3	7.0–7.2	7.5–7.6
DMG-MINO	0.5	0.25	0.25
DMG-DMDOT	0.5	0.12	0.12
Tetracycline	0.5	0.5	1.0
Doxycycline	0.06	0.12	0.25
Minocycline	0.06	0.06	0.06

Possibly some resistant strains contained resistance factors in addition to *tet*(M). Transconjugants or genetically resistant strains of ureaplasmas were not available to assess such possibilities. The step mutants of *M. arginini* selected for tetracycline resistance (Table 3) showed significantly increased resistance to the glycylcyclines only at the third mutation step, and they were 16- to 32-fold more susceptible to the glycylcyclines than to tetracycline and 8-fold more susceptible to minocycline.

Susceptibility testing of mycoplasmas poses difficulties because mycoplasmas grow slowly and do not produce turbidity in broth cultures. This leads to difficulties in determining inoculum size (9, 16, 20) and timing of endpoints. The range of MIC_{50}s reported for *M. pneumoniae* is exceptionally wide, at 0.25 to 3.12 μg/ml for tetracycline and 0.05 to 1.6 μg/ml for doxycycline and minocycline (2, 5, 10, 19, 20, 22, 27, 30, 32). In our study, *M. pneumoniae* was susceptible to tetracycline at 1.0 μg/ml (MIC_{50}) and was susceptible to doxycycline, minocycline, and the glycylcyclines at MIC_{50}s of 0.25 to 0.5 μg/ml (Table 1). The published MIC_{50}s for susceptible *M. hominis* strains are less variable at 0.06 to 0.25 μg/ml for doxycycline and minocycline and 0.12 to 0.5 μg/ml for tetracycline (4–7, 18–20, 22, 27, 30). Our results for *M. hominis* were 1.0 μg/ml for tetracycline and 0.12 to 0.25 μg/ml for doxycycline, minocycline, and the glycylcyclines. For tetracycline-susceptible ureaplasmas, the MICs reported ranged from 0.06 to 0.25 μg/ml for minocycline and doxycycline and from 0.12 to 2 μg/ml for tetracycline (4–6, 20, 22, 26, 27, 30, 31). In the present study, ureaplasmal susceptibilities were 1.0 μg/ml for tetracycline, 0.5 μg/ml for minocycline, and 0.25 μg/ml for doxycycline. It was possible to assess the reproducibility of the agar dilution method by comparing these tetracycline results with those of our previous studies (12, 13). The results were not identical, but the maximum variation in MIC_{50} or MIC_{90} for the three organisms was one twofold dilution.

Overall, *M. pneumoniae*, *M. hominis*, and *U. urealyticum* required MIC_{50}s of 1 μg/ml for tetracycline and were two- to eightfold more susceptible to doxycycline and minocycline. The glycylcyclines are two- to fourfold more active than tetracycline against *M. pneumoniae* and *M. hominis*, a result similar to that reported for gram-positive bacteria (8, 29, 32). However, the glycylcyclines were no more active against tetracycline-susceptible *U. urealyticum* strains than tetracycline. In general, *U. urealyticum* appears to be the least susceptible of these three species to quinolones (15) and is far less susceptible to erythromycin than is *M. pneumoniae* (12, 16, 27).

The increasing resistance of *M. hominis* and *U. urealyticum* to tetracycline leads to therapeutic problems. Koutsky et al. (18) in 1983 concluded that "tetracycline can no longer be considered the drug of choice for treatment of all *M. hominis* infections." Since the distribution of resistance and susceptibility in *M. hominis* clinical isolates toward tetracyclines is strongly bimodal, with no overlap between susceptible and resistant strains as seen in a comparison of Tables 1 and 2 and elsewhere (5, 18), testing for susceptibility at 8 to 16 μg/ml might be sufficient to identify resistant strains. If genetic testing for *tet*(M) becomes available at low cost, direct identification of resistant strains may be possible (6). Tetracycline resistance in *M. pneumoniae* has not been reported, but the broad distribution of tetracycline resistance determinants in bacteria (28) suggests such a possibility. The findings that the glycylcyclines are active against tetracycline-resistant *M. hominis* strains suggest that these compounds have bright promise for treatment of mycoplasmal infections if appropriate pharmacokinetic and safety parameters can be established. Initial studies of the effectiveness of glycylcyclines in mice show promise for the treatment of tetracycline-resistant bacterial infections (29).

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