IN VITRO AND *IN VIVO* CHARACTERIZATION OF NOVEL 8-METHOXY DERIVATIVES OF CHLORTETRACYCLINE

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The *in vitro* activities of three new 8-methoxychlortetracyclines, Sch 36969, 33256 and 34164 were compared to tetracycline, minocycline and doxycycline. Against aerobic Gram-negative rods Sch 36969 had a geometric mean MIC (GMM) of 4.2 μg/ml, about 8-fold more potent than Sch 33256, and similar to all the other compounds. Sch 36969 also had good activity against methicillin-resistant (GMM, 0.21 μg/ml) and -susceptible Staphylococci (GMM, 0.14 μg/ml), Streptococci (GMM, 0.06 μg/ml), and most anaerobic bacteria (GMM, <0.5 μg/ml). In general, Sch 36969 was similar to, or more potent than, all the other compounds tested. Serum levels of Sch 36969 in squirrel monkeys were 4-fold lower (AUC, 4.5 μg·hours/ml) than those of chlortetracycline (AUC, 16.1 μg·hours/ml). In mouse protection tests (PD_{50}s) against various strains of bacteria, Sch 36969 was similar in activity to tetracycline, but up to 6-fold less active than chlortetracycline. The structure activity relationships for these new chlortetracyclines are described.

Three novel derivatives of chlortetracycline (CTC) were isolated from three new strains of Actinomycetales: Sch 33256 from *Actinomadura brunnea* ATCC 39216[1], Sch 36969 from a mutant strain of *A. brunnea*[2], and Sch 34164 from *Micromonospora vesca* ATCC 39499[3]. In this paper the *in vitro* antibacterial activity of these three compounds is compared to tetracycline (TC), doxycycline (DOX), and minocycline (MIN), and the *in vivo* activity of Sch 36969 is compared to CTC and TC[4].

Materials and Methods

Antibiotics

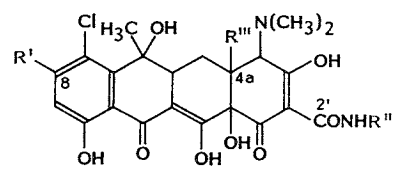
Sch 36969, 33256 and 34164 are 8-methoxy derivatives of CTC. The structural differences between them and CTC are shown in Table 1. These three novel tetracyclines differ from each other in the nature of the substituents at the 2′ and 4a positions.

Other antibiotics used in this study included tetracycline, doxycycline, minocycline (Sigma Chemical Co., St. Louis, MO) and CTC (U.S.P.C., Inc., Rockville, MD).

MIC Determinations

MICs were determined against 76 Gram-negative aerobic bacteria, 91 methicillin-resistant and methicillin-susceptible Staphylococci, and 37 Streptococci by the microtiter method[5]. MICs for *Neisseria gonorrhoeae* (13 strains), *Haemophilus influenzae* (19 strains), and anaerobic bacteria were determined by agar dilution[5].

Table 1. Structures of Sch 36969, 33256 and 34164.



Compound	Substituent at position		
	R′	R″	R‴
Sch 36969	OCH_3	H	H
Sch 33256	OCH_3	CH_3	H
Sch 34164	OCH_3	H	OH
Chlortetracycline	H	H	H

Serum Levels

Serum levels were determined in 6 squirrel monkeys (*ca.* 1 kg) in a crossover fashion. Monkeys were fasted for 18 hours prior to receiving a single oral dose (20 mg/kg) of antibiotic using a nasogastric feeding tube. Blood samples were taken from the jugular vein at various times and serum samples were assayed for antibiotic activity using a microbiological assay with *Bacillus subtilis* ATCC 6633 as the test organism. Areas under the curves (AUCs) were obtained from serum levels vs. time data by the trapezoidal rule[6].

Toxicity (LD_{50}s)

Various doses of the antibiotics were administered intravenously (tail vein) to white male CF1 mice (Harlan Sprague Dawley, Inc., Indianapolis, IN) weighing approximately 20 g. The 50% lethal dose (LD_{50}) for each antibiotic was determined by survival after 96 hours.

Protection Studies (PD_{50}s)

The *in vivo* antibacterial activities of the compounds were determined in male CF1 mice (*ca.* 20 g). Groups of 10 mice were infected intraperitoneally with either *Escherichia coli*, *Klebsiella pneumoniae*, or *Staphylococcus aureus*, and were treated with a single oral dose 30 minutes after infection. The 50% protective dose (PD_{50}) values were estimated by probit analysis based on survivors 7 days post-infection[7].

Results

In Vitro Antibacterial Activities

The *in vitro* activities of Sch 36969, 33256, 34164 and related compounds against aerobic, Gram-negative bacilli are shown in Table 2. The strains were categorized as TC-susceptible (MIC$\leq$4 μg/ml, 23 strains) or TC-resistant (MIC$\geq$8 μg/ml, 53 strains). Against the TC-susceptible strains, Sch 36969 had a geometric mean MIC (GMM) of 4.2 μg/ml, similar to those obtained with Sch 34164, TC, DOX and MIN. It was about 8-fold more potent than Sch 33256 (GMM, 33.0 μg/ml). None of the compounds tested had activity against the TC-resistant strains. Therefore, the rest of the *in vitro* results presented are limited to TC-susceptible strains.

The *in vitro* MIC results against methicillin-resistant ($Meth^R$) and methicillin-susceptible ($Meth^S$) Staphylococci are shown in Table 3. Sch 36969 was the most active compound against $Meth^R$ and

Table 2. MICs of 8-methoxychlortetracyclines against Gram-negative aerobic bacteria.

Strains	No.	Geometric mean MICs (μg/ml, 24 hours, MHB, microtiter)					
		Sch 36969	Sch 33256	Sch 34164	TC	DOX	MIN
TC-susceptible[a]	23	4.2	33.0	7.8	2.3	3.4	5.2
TC-resistant[b]	53	$\geq$81.0	$\geq$121.5	$\geq$88.8	$\geq$46.2	$\geq$35.1	$\geq$22.2

[a] Includes *Escherichia coli* (9), *Klebsiella* sp. (8), *Enterobacter* sp. (4) and *Salmonella-Shigella* sp. (2).

[b] Includes *Escherichia coli* (4), *Klebsiella* sp. (4), *Enterobacter* sp. (1), *Morganella* sp. (1), *Providencia* sp. (10), *Salmonella-Shigella* sp. (4), *Serratia* sp. (9) *Pseudomonas* sp. (20). and

MHB: Mueller-Hinton broth.

Table 3. MICs of 8-methoxychlortetracyclines against Staphylococci.

Strains	No.	Geometric mean MICs (μg/ml, 24 hours, MHB, microtiter)					
		Sch 36969	Sch 33256	Sch 34164	TC	DOX	MIN
Methicillin-resistant	9	0.21	0.86	5.4	0.79	0.43	1.2
Methicillin-susceptible	54	0.14	0.48	2.8	0.48	0.36	1.6

MHB: Mueller-Hinton broth.

Table 4. MICs of 8-methoxychlortetracyclines against Streptococci.

Strains	No.	Geometric mean MICs (μg/ml, 24 hours, MHB+5% horse serum, microtiter)					
		Sch 36969	Sch 33256	Sch 34164	TC	DOX	MIN
Streptococcus pneumoniae	5	0.04	0.07	0.12	0.14	0.12	2.0
S. viridans	2	0.12	0.25	0.71	0.71	0.50	4.0
Group A	5	0.05	0.14	0.29	0.29	0.25	1.3
Group B	1	0.12	0.25	1.0	1.0	0.50	2.0
Group C	3	0.04	0.12	0.25	0.32	0.25	2.0
Group G	3	0.08	0.12	0.40	0.50	0.20	2.5
S. faecalis	2	0.12	0.35	5.7	0.71	0.50	1.4
S. faecium	4	0.06	0.25	1.2	0.50	0.30	2.0
All Streptococci	25	0.06	0.15	0.45	0.36	0.25	1.9

MHB: Mueller-Hinton broth.

Table 5. MICs of 8-methoxychlortetracyclines against anaerobic bacteria.

Strains	No.	Geometric mean MICs (μg/ml, 48 hours, MHA +5% sheep blood)					
		Sch 36969	Sch 33256	Sch 34164	TC	DOX	MIN
Bacteroides fragilis group	16	0.16	0.37	0.46	0.74	0.22	0.10
Other Bacteroides	8	0.12	0.25	0.30	0.32	0.16	0.07
Clostridium difficile	5	0.14	0.05	0.38	0.25	0.08	0.08
C. perfringens	3	0.12	0.20	0.40	0.63	0.20	0.06
Other Clostridia	6	0.28	1.3	0.71	0.50	0.71	0.35
Fusobacterium sp.	3	2.5	40.3	2.5	2.0	4.0	1.3
Other anaerobes	10	0.31	1.2	1.2	0.81	0.71	0.41

MHA: Mueller-Hinton agar.

Table 6. MICs of 8-methoxychlortetracyclines against *Haemophilus influenzae* and *Neisseria gonorrhoeae*.

Strains	No.	Geometric mean MICs (μg/ml, 24 hours, chocolate agar+1% isovitalex)			
		Sch 36969	Sch 33256	Sch 34164	TC
H. influenzae	19	2.5	8.6	1.4	1.2
N. gonorrhoeae	13	0.43	1.3	0.29	0.73

Meth^S strains with GMM values of 0.21 and 0.14 μg/ml, respectively. Sch 33256, TC and DOX all had similar GMM values ranging from 0.36 to 0.86 μg/ml, while MIN had values greater than 1.0 μg/ml. Sch 34164 was least active against Staphylococci with GMM values of 2.8 and 5.4 μg/ml.

The *in vitro* GMM values of the compounds against Streptococci are shown in Table 4. Sch 36969, 33256 and 34164 had good activities against each of the streptococcal groups. However, Sch 34164 was less active against *Streptococcus faecalis* (GMM, 5.7 μg/ml). Sch 36969 had excellent activity with a GMM of 0.06 μg/ml. The GMM values of Sch 33256, 34164, TC and DOX ranged between 0.15 and 0.45 μg/ml, while that of MIN was 1.9 μg/ml.

The *in vitro* GMM values against Gram-negative and Gram-positive anaerobic bacteria, including strains of *Bacteroides*, *Clostridium*, *Fusobacterium*, *Peptococcus* and *Veillonella* are shown in Table 5. All of the compounds were active against all these strains (GMM, $\leq$1.3 μg/ml) with the exception of *Fusobacterium* (GMM, 1.3 to 40.3 μg/ml).

Fig. 1. Serum levels of Sch 36969 and CTC in squirrel monkeys.
○ Sch 36969 (AUC, 4.5 μg·hours/ml), ● CTC (AUC, 16.1 μg·hours/ml).

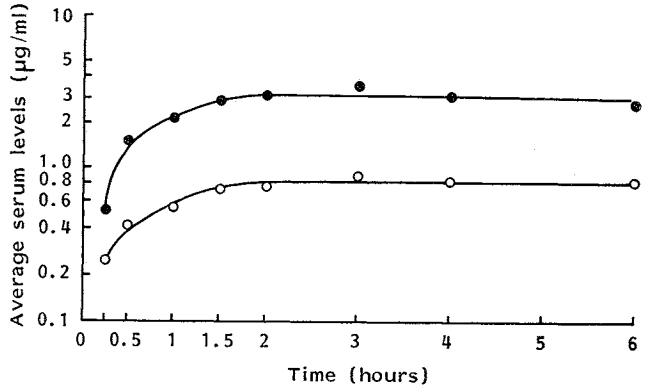


Table 7. *In vivo* antibacterial activity of Sch 36969 oral PD_{50}s (mice, 7 days).

Strain	PD_{50} (mg/kg)		
	Sch 36969	CTC	TC
Staphylococcus aureus			
Strain 502	0.8	0.2	1.0
Strain 006	12.5	2.0	10.0
Strain 835	10.5	7.5	10.5
Escherichia coli 505	15.0	3.0	15.0
Klebsiella pneumoniae 502	225.0	75.0	225.0

Sch 36969, 33256, 34164 and TC had similar activities against *Neisseria gonorrhoeae* (GMM, 0.29 to 1.3 μg/ml, Table 6). Against *Haemophilus influenzae*, Sch 36969, 34164 and TC had equivalent activity (GMM, 1.2 to 2.5 μg/ml), while Sch 33256 was 4~8 times less potent (GMM, 8.6 μg/ml).

Toxicity in Mice

The intravenous LD_{50} values of Sch 36969, 33256 and 34164 in mice were 85, 90 and 110 mg/kg, respectively, similar to that of CTC (110 mg/kg).

Serum Levels in Squirrel Monkeys

Based on its superior *in vitro* activity, Sch 36969 was chosen for further *in vivo* studies. Serum levels of Sch 36969 and CTC were determined in squirrel monkeys. The results are shown in Fig. 1. Two hours after oral dosing, CTC reached a peak of 3 μg/ml, while the peak observed for Sch 36969 was 4-fold lower (0.8 μg/ml). Peak levels for both compounds were maintained for at least 6 hours. Overall, serum levels reflected the peak differences with those of CTC being 4-fold higher (AUC, 16.1 μg·hours/ml) than those of Sch 36969 (AUC, 4.5 μg·hours/ml).

Mouse Protection Tests

The *in vivo* antibacterial activity of Sch 36969 was compared to those of CTC and TC against two Gram-negative and three Gram-positive strains, and the results are shown in Table 7. Against the 3 strains of *S. aureus* tested, PD_{50} values for Sch 36969 and TC ranged from 0.8 to 12.5 mg/kg. PD_{50} values for CTC were lower, ranging from 0.2 to 7.5 mg/kg. Against one strain each of *E. coli* and *K. pneumoniae*, Sch 36969 and TC were similar in activity (PD_{50}s 15.0 and 225.0 mg/kg, respectively). CTC was again 4-fold more active (PD_{50}s, 3.0 and 75.0 mg/kg, respectively).

Discussion

Significant differences were seen in the *in vitro* activities of Sch 36969, 33256 and 34164. Sch 36969 was the most active derivative against most of the bacterial groups tested. It was especially potent against $Meth^R$ (GMM, 0.21 μg/ml) and $Meth^S$ (GMM, 0.14 μg/ml) Staphylococci, Streptococci (GMM, 0.06 μg/ml), and anaerobic bacteria (GMM, <0.5 μg/ml). Sch 36969 was also consistently equal to, or more potent than, TC. The *in vitro* differences between the three 8-methoxychlortetracyclines can be correlated with the differences in their chemical structures. For example, results indicate that the presence of the methyl group in the 2′-*N* position reduced the potency of Sch 33256 against aerobic Gram-negative rods (GMM, 33.0 μg/ml). Similar results have been reported for other 2′-*N* derivatives[8]. Although the poor potency of Sch 33256 was also observed against *H. influenzae* (GMM, 8.6 μg/ml), this was not the case against anaerobic Gram-negative bacilli. Sch 33256 had good activity against *Bacteroides fragilis* (GMM, 0.37 μg/ml), similar to the other two derivatives.

The reduced potency of Sch 34164 against $Meth^R$ (GMM, 5.4 μg/ml) and $Meth^S$ (GMM, 2.8 μg/ml) Staphylococci must be due to the presence of the hydroxyl group in the 4a position since this is the only difference between it and Sch 36969.

Structural variations may also account for the observed *in vivo* differences between Sch 36969 and CTC. The presence of the 8-methoxy group on Sch 36969 may have interfered with its absorption in squirrel monkeys, resulting in 4-fold lower serum levels. Although serum levels were not determined in mice, the data obtained in monkeys suggest that the reduced efficacy observed in mice may also be due to lower serum levels.

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