BIOLOGICAL PROPERTIES OF NEW ACYL DERIVATIVES OF TYLOSIN

ROKURO OKAMOTO, MASAMI TSUCHIYA, HIDEO NOMURA, HIROSHI IGUCHI, KOHKI KIYOSHIMA, SENJI HORI and TAIJI INUI

Central Research Laboratories, Sanraku Ocean Co., Ltd.,
9–1, Johnan 4 chome, Fujisawa, 251 Japan

TSUTOMU SAWA, TOMIO TAKEUCHI and HAMAO UMEZAWA

Institute of Microbial Chemistry,
Kamiosaki, Shinagawa-ku, Tokyo, 141 Japan

(Received for publication August 1, 1980)

The antibacterial activity of acyl derivatives of tylosin were examined *in vitro* and *in vivo*. The 4''-acyl group in the acylated tylosins enhanced the antibacterial activity and antimycoplasmal activity against some macrolide-resistant strains. The orally administered 3-acetyl-4''-isovaleryltylosin produced a higher blood level in mice and rabbits than tylosin and a good therapeutic effect on the infection of *Staphylococcus aureus* Smith in mice.

In a previous paper,[1] the preparation of 3- and/or 4''-acyl derivatives of tylosin by microbial transformation and their physico-chemical properties were described. The improved antimicrobial activity of 4''-acylated tylosins against macrolide-resistant clinical isolates of *Staphylococcus aureus* and *Streptococcus pyogenes* was briefly reported.[2]

Depending on the structure of aglycone, 16-membered macrolide antibiotics can be classified into the carbomycin-leucomycin subgroup and the tylosin-chalcomycin subgroup.[3] It is well known that the substituents at positions 3, 2' and 4'' of 16-membered macrolide antibiotics are involved in the degree of the antibacterial activities and the pharmacological properties. For example, ŌMURA *et al.* reported that 3-acetylleucomycins were superior to 3-hydroxyleucomycins in the blood level after oral administration, but inferior in the *in vitro* antibacterial activity; the antibacterial activity of 4''-acylleucomycins was elevated in the increasing order of acetyl, propionyl, butyryl and isovaleryl; 2'-acylated leucomycins were antimicrobially inactive.[3,4]

In the present paper, the antimicrobial activities of acylated tylosins were compared with those of acyl derivatives of angolamycin, spiramycin, leucomycin and carbomycin, with particular reference to the position of acylation. The biological properties of 3-acetyl-4''-isovaleryltylosin which seem most promising from the viewpoints of antimicrobial activity were examined in comparison with those of tylosin.

Materials and Methods

Antibiotics

Tylosin, angolamycin and spiramycin were converted to 3-acetyl- and/or 4''-isovaleryl derivatives by using culture broths or washed mycelia of a high-acylase mutant (strain No. 8254) of *Streptomyces thermotolerans* ATCC 11416.[1] Tylosin was also chemically acylated at the desired position(s) with acetyl chloride and/or isovaleryl chloride after preliminary masking of the other functional (hydroxyl) group(s) with monochloroacetyl chloride. Chemical acylation of tylosin and the microbial acylation of angolamycin and spiramycin will be reported elsewhere.

Leucomycins U and A_3, oxytetracycline, erythromycin, chloramphenicol, spiramycin and tylosin were purified from commercially available preparations. Carbomycin A and angolamycin were prepared by culture of *Streptomyces halstedii* NRRL B-2331 and *Streptomyces erythermus* ATCC 14975 respectively. Deltamycin X (4''-deacylcarbomycin A) was prepared by 4''-deacylation of carbomycin A with *Bacillus megaterium* IAM 1032.[5,6]

Susceptibility tests

(1) Antibacterial activity

MIC (minimal inhibitory concentrations) were obtained by the two-fold serial agar dilution method in BHIA (brain heart infusion agar) (Eiken Chemical Co., Ltd. Tokyo) supplemented with 10% horse blood for *Streptococcus pyogenes* and in BHIA for the other test microbes. One loopful of an overnight culture of the test organisms in BHI medium was streaked on assay plates containing the indicated concentration of test materials and the plates were incubated at 37°C for 20 hours. Macrolide-resistant clinical isolates of *Staphylococcus aureus* and *Streptococcus pyogenes* were supplied by Prof. S. MITSUHASHI, Department of Microbiology, School of Medicine, Gunma University.

(2) Antimycoplasmal activity

MIC against *Mycoplasma gallisepticum* were measured by the serial tube dilution method in the medium containing 17.3 g of dehydrated PPLO enrichment broth (Eiken Chemical Co., Ltd.), 150 ml fresh horse serum (Japan Biotest Co., Ltd., Tokyo), 1,000,000 units potassium benzylpenicillin and 2 mg phenol red (pH 7.8 before autoclaving) per liter.

Serial two-fold dilutions of an antibiotic in the above medium were inoculated with a tenth volume of the seed culture of *Mycoplasma* ($10^5 \sim 10^6$ colony-forming units/ml) and then incubated at 37°C for 3 ~ 5 days.

Strains of *Mycoplasma gallisepticum* employed in the present experiments were supplied by Dr. C. KUNIYASU, National Institute of Animal Health, Kokubunji, Japan.

Stability in artificial gastric juice

A material was dissolved at a concentration of 1,000 μg/ml in a gastric juice composed of 2.0 g sodium chloride, 3.2 g pepsin and dilute hydrochloric acid in 1,000 ml of distilled water (pH 1.2), and the solution was incubated at 37°C for the indicated periods of time. The antibiotic remaining in the solution was extracted with ethyl acetate at pH 9.0 and then chromatographed on a silicagel thin-layer plate. The amount of the macrolide antibiotic was densitometrically assayed. It was also disc-assayed with *Bacillus subtilis* ATCC 6633.

Blood levels

A single dose of 50, 100 or 200 mg/kg of the drug was orally administered to 5 week-old male *ddY* mice (20±1 g) (Shizuoka Agricultural Cooperative Association for Experimental Animal, Shizuoka) and 8 month-old male white rabbits (3.0±0.1 kg) (Ichikawaya, Tokyo) which had been fasted overnight.

Mouse blood samples were withdrawn from the retroorbital sinus by heparinised capillary tubes at the indicated times and the tubes were centrifuged at 3,000×*g* for 5 minutes to yield plasma. Rabbit blood samples were collected from the auricular vein with a heparinised syringe at the indicated times and centrifuged at 3,000×*g* for 5 minutes. Concentrations of the antibiotic in plasma were bioassayed by the paper disk method with *Sarcina lutea* ATCC 9341.

Therapeutic effect

Sixteen hour-old culture of *Staphylococcus aureus* Smith (pathogen) in heart infusion broth (Eiken Chemical Co., Ltd.) was diluted 5-fold in the same medium and then mixed at 1: 1 with 10% hog gastric mucin (Tokyo Chemical Industry Co., Ltd., Tokyo). One half milliliter of the suspension was intraperitoneally injected into 5 week-old *ddY* mice (male, 20±1 g). A suspension (0.25 ml) of a test antibiotic in 0.5% sodiumcarboxymethylcellulose (Daiichi Pure Chemical Co., Ltd., Tokyo) was given orally to the experimental groups of 6 ~ 10 mice each singly at 1 hour or at the indicated intervals after injection. The survival rate was calculated at 5 days after treatment.

Results and Discussion

Antimicrobial Significance of the 4''-Acyl Group in the Tylosin-Chalcomycin Subgroup

MIC of acylated tylosins against *Staphylococcus aureus* and *Streptococcus pyogenes* were compared with those of acylated angolamycins, acylated spiramycins, leucomycins and carbomycins (Table 1). *Staphylococcus aureus* Smith and *Streptococcus pyogenes* NY-5 were sensitive to all the compounds tested. When the erythromycin-resistant clinical isolates of *Staphylococcus aureus* (MS 8710 and MS 9937) and *Streptococcus pyogenes* (MH 604 and MH 771) were tested, 4''-isovaleryltylosins (**3** and **4**) and 4''-isovalerylangolamycins (**7** and **8**) were found to have improved MIC in comparison with the others. The 3-acetyl group seems not to be important for improvement of the antibacterial activity, because 3-acetyltylosin (**2**) and 3-acetylangolamycin (**6**) were inactive against these resistant strains as well as tylosin (**1**) and angolamycin (**5**).

In contrast, the improvement of the antibacterial activity due to 4''-acylation was not observed in spiramycins, leucomycins and carbomycins all of which belonged to the carbomycin-leucomycin subgroup. It is interesting to note that the 4''-acyl group gives a different effect between tylosin-chalcomycin subgroup and carbomycin-leucomycin subgroup.

Comparative Antibacterial Effects of the 3-, 2'-, 4''- and 4'''-Acyl Groups of Tylosin on MIC

The tylosin-chalcomycin subgroup is structurally different from the carbomycin-leucomycin subgroup in the presence of a third sugar moiety (mycinose) attached through the methylene chain to posi-

Table 1. Effects of the 3-acetyl and 4''-isovaleryl groups of 16-membered macrolides on MIC against Gram-positive bacteria.

Compound	MIC (μg/ml)					
	Staphylococcus aureus			*Streptococcus pyogenes*		
	Smith	MS 8710	MS 9937	NY 5	MH 604	MH 771
1. Tylosin	0.39	>200	100	0.39	>200	100
2. 3-Acetyltylosin	0.39	>200	100	0.39	200	100
3. 4''-Isovaleryltylosin	0.39	50	12.5	0.39	12.5	3.13
4. 3-Acetyl-4''-isovaleryltylosin	0.39	25	12.5	0.39	12.5	3.13
5. Angolamycin	0.39	>200	200	0.39	>200	100
6. 3-Acetylangolamycin	0.39	>200	200	0.39	>200	100
7. 4''-Isovalerylangolamycin	0.39	25	12.5	0.39	25	3.13
8. 3-Acetyl-4''-isovalerylangolamycin	0.39	50	25	0.39	25	3.13
9. Spiramycin	0.39	>200	>200	0.39	>200	>200
10. 3-Acetyl-4''-isovalerylspiramycin	0.39	>200	>200	0.39	>200	>200
11. Leucomycin U	0.39	>200	>200	0.39	>200	>200
12. Leucomycin A_8	0.39	>200	>200	0.39	>200	>200
13. Deltamycin X*	0.81	>200	>200	0.81	>200	>200
14. Carbomycin A	0.39	>200	>200	0.39	>200	>200

MIC were determined by the agar dilution method in BHI medium containing 10 % horse blood.
Resistance pattern, MS 8710: EM, PC-G; MS 9937: EM, OTC; MH 604 and MH 771: EM, PC-G, CEX, OTC (EM=erythromycin, PC-G=benzylpenicillin, OTC=oxytetracycline, CEX=cephalexin).

* Deltamycin X=4''-deisovalerylcarbomycin A.

tion 14 of the aglycone in the former. Among five hydroxyl groups of tylosin, two (positions 3 and 4″) can be acylated by streptomycetes and four (positions 3, 2′, 4″ and 4‴) by chemical treatments under mild reaction conditions (unpublished work).

In order to determine the relative importance of the 3-, 2′-, 4″- and 4‴-acyl groups of tylosin in the antibacterial activity, tylosin was subjected to the selective acylation by chemical process. MIC of the sixteen tylosin derivatives against the sensitive strain and the resistant strain are presented in Table 2.

Based on the results in Table 2, the following may be remarked:

(1) The 3-acetylation neither enhances nor detracts from the antibacterial activity (2 *vs.* 1; 5 *vs.* 3; 6 *vs.* 4; 9 *vs.* 7; 12 *vs.* 10 in Table 2).

(2) The 4″-acylation results in the improved activity against the resistant strain, while no change is caused in the activity against the sensitive strain (4 *vs.* 1; 6 *vs.* 2; 13 *vs.* 2; 9 *vs.* 5).

Table 2. Relative significance of the 3-, 2′-, 4″- and 4‴-acyl groups of tylosin in expression of the antibacterial activity against *Staphylococcus aureus*.

	Tylosin acylated at				MIC (μg/ml)	
					St. aureus	
	C-3	C-2′	C-4″	C-4‴	Smith	MS 8710
1.	OH	OH	OH	OH	0.39	>200
2.	OAc	OH	OH	OH	0.39	>200
3.	OH	OAc	OH	OH	1.56	>200
4.	OH	OH	OAc	OH	0.39	50
5.	OAc	OAc	OH	OH	1.56	>200
6.	OAc	OH	OAc	OH	0.39	25
7.	OH	OAc	OAc	OH	1.56	>200
8.	OH	OAc	OH	OAc	3.13	>200
9.	OAc	OAc	OAc	OH	0.78	>200
10.	OH	OAc	OAc	OAc	3.13	>200
11.	OAc	OH	OAc	OAc	1.56	>200
12.	OAc	OAc	OAc	OAc	3.13	>200
13.	OAc	OH	OiV	OH	0.39	25
14.	OAc	OH	OiV	OiV	3.13	>200
15.	OAc	OiV	OH	OH	1.56	>200
16.	OAc	OH	OH	OiV	3.13	>200

OiV: O-isovaleryl

(3) The 2′- and/or 4‴-acylations lead to the reduction in the antibacterial activity against the sensitive strain (3 *vs.* 1; 8 *vs.* 1; 5 *vs.* 2; 15 *vs.* 2; 16 *vs.* 2; 9 *vs.* 6; 11 *vs.* 6; 12 *vs.* 6; 14 *vs.* 13) and to the elimination of the 4″-acylation dependent effect in the antibacterial activity (7 *vs.* 4; 10 *vs.* 4; 9 *vs.* 6; 11 *vs.* 6; 12 *vs.* 6; 14 *vs.* 13).

In brief, the acylation of 4″-hydroxyl group of tylosin results in the improved antibacterial activity against some macrolide-resistant pathogens, while acylation of the 2′- and/or 4‴-hydroxyl group(s) of tylosin has a negative effect. In contrast to leucomycins, tylosin is not affected in the antibacterial activity by 3-acylation.

Table 3. MIC of 3-acetyl-4″-isovaleryltylosin in comparison with known antibiotics against 34 macrolide-resistant clinical isolates of *Staphylococcus aureus*.

Compound	Cumulative % susceptible at a concentration (μg/ml) of							
	1.56	3.13	6.25	12.5	25	50	100	200
3-Acetyl-4″-isovaleryltylosin	0	21	21	26	79	88	94	100
Tylosin	9	9	26	26	26	26	26	26
Spiramycin	0	0	0	9	9	24	24	24
Oxytetracycline	0	0	24	29	29	29	29	29
Leucomycin A_3	9	21	21	21	21	21	21	24
Erythromycin	0	0	0	0	0	0	0	0
Chloramphenicol	0	0	0	24	29	79	91	100

MIC were determined by the agar dilution method (5×10^8 cells/ml). Range of drug concentrations tested: 0.19~200 μg/ml.

Antibacterial Activity of 3-Acetyl-4''-Isovaleryltylosin and Other Antibiotics

Among 34 erythromycin-resistant strains of *Staphylococcus aureus*, 88% were inhibited by less than 50 μg/ml of 3-acetyl-4''-isovaleryltylosin, 94% by 100 μg/ml and 100% by 200 μg/ml, while only 30% were inhibited by 200 μg/ml of the other macrolides and oxytetracycline. Chloramphenicol was as active as 3-acetyl-4''-isovaleryltylosin (Table 3). Thus the improvement of antibacterial activity of tylosin against macrolide-resistant *Staphylococci* due to the 4''-acylation was also shown in the activity against clinical isolates.

Antimycoplasmal Activity

Tylosin is currently used for the treatment of mycoplasma infections in animals. The antimycoplasmal activity of 3-acetyl-4''-isovaleryltylosin and the reference antibiotics was examined against a sensitive strain (KP-13) and the macrolide-resistant strains (E-5, E-11, A-68 and A-72) of *Mycoplasma gallisepticum* (Table 4).

The effect of 4''-acylation enhanced the antimycoplasmal activity more markedly than the antibacterial activity.

Table 4. Antimycoplasmal activity of 3-acetyl-4''-isovaleryltylosin in comparison with known antibiotics against *Mycoplasma gallisepticum*.

Compound	MIC (μg/ml)				
	Mycoplasma gallisepticum				
	E-5	E-11	A-68	A-72	KP-13
3-Acetyl-4''-isovaleryltylosin	0.62	0.31	0.31	0.31	0.02
Tylosin	10	5	2.5	2.5	0.02
Erythromycin	100	100	100	100	0.1
Spiramycin	100	100	100	100	0.1
Chloramphenicol	10	2.5	25	10	25
Oxytetracycline	0.62	0.31	1.3	0.62	0.62

MIC were determined by the tube dilution method ($10^5 \sim 10^6$ CFU/ml).

Table 5. Stability of 3-acetyl-4''-isovaleryltylosin in artificial gastric juice.

Compound	Incubation (hours)	Amount of antibiotic remaining	
		t.l.c. assay (%)	Bio-assay (mm)
Tylosin	0	100	17.7
	2	t*	17.0
	4	t	17.0
	6	t	15.4
3-Acetyl-4''-isovaleryltylosin	0	100	16.5
	2	70	16.5
	4	38	15.9
	6	13	15.3
Leucomycin A_3	0	100	15.8
	2	58	15.6
	4	34	12.4
	6	13	8.1

* t=trace

Drug was incubated at a concentration of 1,000 μg/ml for the indicated periods of time at 37°C. Amount of the antibiotic remaining was measured by thin-layer chromatography (t.l.c.) and by the disc assay with *Bacillus subtilis* ATCC 6633.

Stability in Artificial Gastric Juice

Since most macrolide antibiotics are often administered orally in the treatment of infections, the stability at a pH as low as 1.0 is important. Therefore, the stability of 3-acetyl-4''-isovaleryltylosin in artificial gastric juice (pH 1.2) was examined, testing the activity by bioassay and TLC methods (Table 5). Tylosin was rapidly decomposed to give desmycosin which was still bioactive.[7] Leucomycin A_3 and 3-acetyl-4''-isovaleryltylosin, on the other hand, were slowly transformed to demycarosylleucomycin and 3-acetyldesmycosin respectively. Thus, 3-acetyl-4''-isovaleryltylosin was shown to be more stable in the stomach than tylosin.

Blood Levels of Tylosin and Acylated Tylosins after Oral Administration in Mice and Rabbits

Tylosin is described to give poor blood levels after oral administration.[8] One of the purposes of its acylation is to obtain a derivative which can give a high blood level. After a single dose of tylosin, 3-acetyltylosin or 3-acetyl-4''-isovaleryltylosin was orally given to mice and rabbits, the blood samples

Table 6. Blood levels of 3-acetyl-4''-isovaleryltylosin after oral administration into mice and rabbits.

	Compounds	Dose (mg/kg)	Blood level (μg/ml)				
			15 min.	30 min.	1 hour	2 hours	3 hours
Mouse	Tylosin	100		t	0.45	t	
	3-Acetyltylosin	100		t	0.5	t	
	3-Acetyl-4''-isovaleryltylosin	100		9.5	14.0	2.5	1.8
		200	14.7	23.2	13.7	7.5	
Rabbit	Tylosin	50	t				
		200		0.4	t		
	3-Acetyl-4''-isovaleryltylosin	50		5.2	1.9	1.0	t
		200		21.3	18.5	6.2	3.6

Concentrations of the drug in blood samples were disc-assayed with *Sarcina lutea* ATCC 9341.
t=trace.

Table 7. Therapeutic effects of orally given 3-acetyl-4''-isovaleryltylosin and tylosin in mice infected systemically with *Staphylococcus aureus* Smith.

	Compound	Dose (mg/kg)	Survived/Treated				
			Day 1	Day 2	Day 3	Day 4	Day 5
Experiment 1	Tylosin	400	6/10				6/10
		300	6/10	5/10			5/10
		200	4/10	3/10			3/10
		100	3/10	1/10			1/10
	3-Acetyl-4''-isovaleryltylosin	400					10/10
		300	7/10				7/10
		200	4/10	3/10			3/10
		100	1/10				1/10
	Untreated		0/10				0/10
Experiment 2	Tylosin	400×4					5/5
		100×4	1/5	0/5			0/5
		25×4	0/5				0/5
	3-Acetyl-4''-isovaleryltylosin	400×4					5/5
		100×4					5/5
		25×4		3/5	2/5		2/5
	Untreated		0/5				0/5

Experiment 1: Challenge dose $50\times LD_{50}$ in 5% mucin, i.p.; treatment 1 hour after infection.
Experiment 2: Challenge dose $20\times LD_{50}$ in 5% mucin, i.p.; treatment 1, 4, 8 and 24 hours after infection.

were collected at the indicated times and the concentrations of the antibiotics were bioassayed with *Sarcina lutea* ATCC 9341 (Table 6).

In mice, 3-acetyl-4''-isovaleryltylosin provided a markedly higher level than tylosin and 3-acetyltylosin. This was more clearly confirmed in rabbits. Since 3-acetyltylosin was as poor as tylosin, the improved blood level with 3-acetyl-4''-isovaleryltylosin could be ascribed to 4''-isovalerylation.

Treatment of Experimental Infections with *Staphylococcus aureus* Smith

Therapeutic effect of orally given 3-acetyl-4''-isovaleryltylosin was examined on mice systemically infected with *Staphylococcus aureus* Smith (Table 7). It is apparent that 3-acetyl-4''-isovaleryltylosin is superior to tylosin in the *in vivo* effect. This agrees with the improved blood levels. Multiple doses of 3-acetyl-4''-isovaleryltylosin showed a good effect in preventing the infection.

References

1) OKAMOTO, R.; T. FUKUMOTO, H. NOMURA, K. KIYOSHIMA, K. NAKAMURA, A. TAKAMATSU, H. NAGANAWA, T. TAKEUCHI & H. UMEZAWA: Physico-chemical properties of new acyl derivatives of tylosin produced by microbial transformation. J. Antibiotics 33: 1300~1308, 1980
2) OKAMOTO, R.; H. NOMURA, M. TSUCHIYA, H. TSUNEKAWA, T. FUKUMOTO, T. INUI, T. SAWA, T. TAKEUCHI & H. UMEZAWA: The activity of 4''-acylated tylosin derivatives against macrolide-resistant Gram-positive bacteria. J. Antibiotics 32: 542~544, 1979
3) ŌMURA, S. & A. NAKAGAWA: Chemical and biological studies on 16-membered macrolide antibiotics. J. Antibiotics 28: 401~403, 1975
4) ŌMURA, S.; M. KATAGIRI, I. UMEZAWA, K. KOMIYAMA, K. SEKIKAWA, A. MATSUMAE & T. HATA: Structural-biological activities relationships among leucomycins and their derivatives. J. Antibiotics 21: 532~538, 1968
5) OKAMURA, K.; A. KOKI, Y. MUTOH, Y. SHIMAUCHI & T. ISHIKURA: Fermentative production of deltamycin and deacyldeltamycin. J. Ferment. Technol. 55: 347~355, 1977
6) OKAMOTO, R.; T. FUKUMOTO, K. IMAFUKU, T. OHKUBO, K. KIYOSHIMA, A. TAKAMATSU & T. TAKEUCHI: Screening for 16-membered macrolide transforming microorganisms. J. Ferment. Technol. 57: 519~528, 1979
7) HAMILL, R. L.; M. E. HANEY, Jr., M. STAMPER & P. F. WILEY: Tylosin, a new antibiotic. II. Isolation, properties and preparation of desmycosin, a microbiologically active degradation product. Antibiot. & Chemoth. 11: 328~334, 1961
8) BERKMAN, R. N.; E. A. RICHARD, R. L. VANDUYN & R. M. KLINE: The pharmacology of tylosin, a new antibiotic, in the chicken. Antimicr. Agents & Chemoth. 1960: 595~604, 1961