

## Pharmacodynamic Interaction between RP 59500 and Gram-Positive Bacteria Infecting Fibrin Clots

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The fibrin clot penetration and in vivo bactericidal activity of RP 59500, a new semisynthetic streptogramin, for two *Staphylococcus aureus* strains (one methicillin resistant and the other methicillin susceptible), two *Staphylococcus epidermidis* strains (one methicillin resistant and the other methicillin susceptible), and one *Enterococcus faecalis* strain were evaluated. The clots, inserted subcutaneously, were infected with a mean of  $10^8$  CFU of the pathogen per g. For each strain, groups of four rabbits received a single intravenous injection of 50 mg of RP 59500 per kg of body weight over 30 min. The mean peak level of RP 59500 in serum in the infected rabbits was  $61.9 \pm 6.3$   $\mu\text{g/ml}$ . The drug was detectable in serum at a level of 0.8  $\mu\text{g/ml}$  up to 4 h after administration. The mean peak fibrin clot drug level at 1 h was  $3.3 \pm 0.1$   $\mu\text{g/g}$ . At 6 h, the level in clots was  $1.2 \pm 0.1$   $\mu\text{g/g}$ . The mean half-life in serum in infected rabbits was  $0.34 \pm 0.01$  h, while in clots the drug exhibited a longer half-life of  $3.8 \pm 0.4$  h. In vivo, this new streptogramin sterilized the clots infected with the two *S. aureus* strains studied in less than 1 h and induced a marked reduction in colony counts of the two *S. epidermidis* strains studied for up to 24 h. The activity of the streptogramin against *E. faecalis* was limited. These results suggest that RP 59500 should be further evaluated for the treatment of infection with methicillin-resistant staphylococci.

RP 59500 belongs to the streptogramin class of antibiotics. It is a mixture of two soluble semisynthetic derivatives of the purified natural pristinaamycin produced by fermentation of the *Streptomyces pristinaespiralis* compounds PI (RP 57669) and PII (RP 54476), which are present in a proportion of 30:70 (wt/wt), respectively (1, 7). The antibiotics of the streptogramin family are inhibitors of protein biosynthesis through their irreversible blocking action on the ribosome. RP 59500 is active against a range of gram-positive pathogenic bacteria. Included in this group are methicillin-susceptible and -resistant *Staphylococcus aureus* and *Staphylococcus epidermidis*, erythromycin-resistant *Streptococcus pneumoniae*, other streptococci, and clostridia. It also has activity against *Neisseria* spp., *Moraxella catarrhalis*, *Haemophilus influenzae*, *Legionella* spp., *Mycoplasma* spp., and chlamydiae (7). As it is the first formulation of an injectable streptogramin, it may, if proven effective, have great clinical importance for the treatment of serious infections caused by gram-positive organisms.

The present investigation was undertaken to study the efficacy and penetration of RP 59500 in fibrin clots. Fibrin is one of the main constituents of the inflammatory process, and bacteria located within the core of fibrin clots are protected from host defenses and the action of the antibiotics, as they often interact with and adhere to major constituents of clots. Moreover, penetration of antimicrobial agents within the clots is quite variable. New antibiotics that can penetrate fibrin and sterilize gram-positive bacteria are needed. The experimental model of the implanted fibrin clot in subcutaneous pockets in rabbits was used to evaluate the pharmacodynamic interaction between RP 59500 and gram-positive bacteria responsible for endocarditis.

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Conference on Antimicrobial Agents and Chemotherapy [16].)

### MATERIALS AND METHODS

**Bacterial strains.** The five strains used for our experiments were originally isolated from patients at the Centre Hospitalier de l'Université Laval. They included methicillin-resistant *S. aureus* STA 664, methicillin-susceptible *S. aureus* STA 560, methicillin-resistant *S. epidermidis* SE 1130, methicillin-susceptible *S. epidermidis* SE 438, and *Enterococcus faecalis* STRF 285. The MICs and MBCs of RP 59500 for these pathogens were determined according to National Committee for Clinical Laboratory Standards recommendations (11). Possible inoculum effects were also investigated with inocula of  $10^3$ ,  $10^5$ , and  $10^7$  CFU/ml.

**Preparation of noninfected and infected fibrin clots.** As previously described (10), a sterile solution of 3% bovine fibrinogen (Sigma Chemical Co., St. Louis, Mo.) was supplemented with 5% Mueller-Hinton broth (sterile or infected with an inoculum of  $10^7$  to  $10^8$  CFU of the strain to be studied per ml) and distributed as 2-ml aliquots into siliconized test tubes (13 by 100 mm); 0.1 ml of bovine thrombin (250 U/ml; Parke-Davis and Co., Detroit, Mich.) was added to each tube. After a 1-h incubation at 37°C, the clots were gently removed, washed in sterile water, and inserted subcutaneously in rabbits. The concentration of protein in fibrin clots reached a maximum of 15% of the protein content in serum.

**Rabbit model.** As described earlier (10), New Zealand White female rabbits (weight, 1.8 to 2.2 kg) were given an intramuscular injection of 20 mg of chlorpromazine per kg of body weight. Both flanks were shaved and swabbed with Povidine. The skin was anesthetized with 2% lidocaine, and a 4-cm-long incision was made on each side. After blunt dissection of the skin, four to six infected or noninfected

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clots were placed in each subcutaneous pocket to limit clustering. Steri-Strip skin closures (3M) were applied to close the incision. A scalp vein needle (23 gauge) inserted in the central vein of the left ear served for infusion of the antibiotic, the steady rate (19 ml/h) of flow being ensured by a Harvard infusion pump. Another scalp vein needle (22 gauge) inserted in the central vein of the right ear was used to collect blood samples. Just before the beginning of the infusion, a 0.3-ml/kg intramuscular injection of ketamin-xylozalin was given.

**Antibiotic regimen.** A dose of 50 mg of RP 59500 per kg was infused over 30 min in each rabbit. Four rabbits were used for each bacterial strain and for experiments in which sterile clots were inserted. For each rabbit, blood and fibrin clots were removed aseptically before the administration of the antibiotic and at 0.08, 0.15, 0.5, 1, 2, 4, 6, and 24 h for serum and 0.5, 1, 2, 4, 6, and 24 h for clots.

**Determination of drug concentration and protein binding.** Clots were weighed and homogenized in 1 volume of trypsin solution (Difco). The concentration of trypsin used to digest the clots did not in any way influence bacterial numeration. Dissolved clots and serum samples were bioassayed by a conventional agar diffusion method with *Sarcina lutea* (*Micrococcus luteus*) ATCC 9341 as the assay organism in Difco antibiotic medium no. 1. Standard solutions were prepared by diluting known amounts of antibiotic in rabbit serum or in trypsinized clots. The mean sensitivity and linearity for serum assays were, respectively,  $0.3 \pm 0.1 \mu\text{g/ml}$  and  $0.998 \pm 0.001$ . The intra-assay coefficients of variation for serum were 23.3% for the high concentration (50  $\mu\text{g/ml}$ ) and 16.6% for the low concentration (1  $\mu\text{g/ml}$ ), while the interassay variations were 3.0% for the high concentration and 8.2% for the low one. The mean sensitivity and linearity for clot assays were, respectively,  $0.4 \pm 0.2 \mu\text{g/ml}$  and  $0.997 \pm 0.001$ . The intra-assay coefficients of variation for clots were, respectively, 29.1% for the high concentration (4  $\mu\text{g/g}$ ) and 20.0% for the low concentration (0.4  $\mu\text{g/g}$ ). As in the case of the interassay variation, we have obtained 3.7% for the high concentration and 8.7% for the low concentration. The degree of protein binding of RP 59500 to rabbit serum was determined for four concentrations, namely, 25, 50, 100, and 250  $\mu\text{g/ml}$ .

**In vivo efficacy.** The efficacy of the drug regimen was evaluated by analysis of the bacterial content of infected clots at each interval as previously described (10). Appropriate dilutions of trypsinized clots were inoculated on agar and then incubated at 37°C for 24 h. Since 0.025-ml samples that were diluted in 1/10 increments from 1- to 100,000-fold were spread over an agar plate (15 by 100 mm), drug carryover was not a problem.

**Pharmacokinetics and statistical analyses.** Pharmacokinetic analyses were done with the pharmacokinetic program Anaphar. A one-compartment model was chosen for both serum and clots. The area under the curve (AUC) of concentration versus time was obtained by the method of successive trapezoidal approximation from time zero to 24 h. The AUC from zero to infinity was calculated by adding the portion  $C_{24\text{h}}/K_e$ , where  $C_{24\text{h}}$  is the concentration at 24 h and  $K_e$  is the slope of the elimination phase (13). The results are presented as the means  $\pm$  the standard errors of the means of 23 experiments. Statistical analyses were performed by using an analysis of variance with repeated measures and a one-way analysis of variance. Statistical evaluations of efficacy data were performed by correlation tests. (Type I error was set at 5%.)

TABLE 1. MICs and MBCs of RP 59500 and inoculum effect

Strain	Inoculum (CFU/ml)	MIC ( $\mu\text{g/ml}$ )	MBC ( $\mu\text{g/ml}$ )
<i>S. aureus</i>	Methicillin susceptible	$10^3$	0.5
		$10^5$	0.5
		$10^7$	1.0
	Methicillin resistant	$10^3$	0.5
		$10^5$	1.0
		$10^7$	1.0
<i>S. epidermidis</i>	Methicillin susceptible	$10^3$	0.25
		$10^5$	0.25
		$10^7$	0.5
	Methicillin resistant	$10^3$	0.25
		$10^5$	0.5
		$10^7$	0.5
<i>E. faecalis</i>	$10^3$	8.0	
	$10^5$	8.0	
	$10^7$	32	

## RESULTS

**In vitro studies.** The respective MICs and MBCs of RP 59500 for the five clinical isolates are shown in Table 1. The mean levels of protein binding to rabbit serum for concentrations of 25, 50, and 100  $\mu\text{g/ml}$  were, respectively, 91.2, 81.3, and 97.5%, with a mean of 90.0% ( $\pm 8.2\%$ ). At a concentration of 250  $\mu\text{g/ml}$ , the binding fell to 71%, suggesting a saturation process.

**RP 59500 pharmacokinetics.** RP 59500 levels after therapy in both serum and fibrin clots of normal and infected animals are shown in Fig. 1. The peak antibiotic concentration in serum ( $61.9 \pm 3 \mu\text{g/ml}$ ) was achieved at 15 min after the beginning of the infusion, and the peak concentration in clots ( $3.1 \pm 0.1 \mu\text{g/g}$ ) was achieved at 1 h. At 4 h, the level of RP 59500 in serum was 0.8  $\mu\text{g/ml}$ , while at 6 h the level in clots was  $1.2 \pm 0.2 \mu\text{g/g}$ . RP 59500 pharmacokinetic parameters are shown in Table 2.

**In vivo bactericidal activity.** The comparative in vivo efficacies of RP 59500 against the five bacterial strains are shown in Fig. 2 to 4. The results are expressed as the mean log differences between the number of CFU at baseline and those recovered at different times afterwards.

In the fibrin clots infected with methicillin-susceptible (Fig. 2a) or methicillin-resistant (Fig. 2b) *S. aureus* in

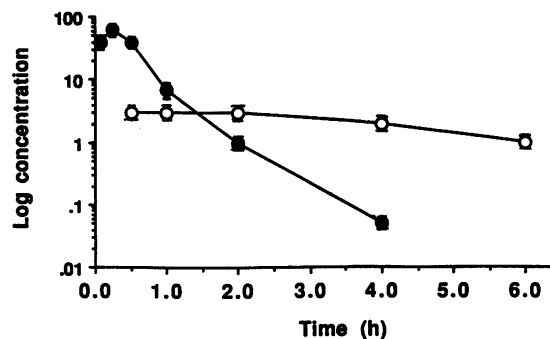


FIG. 1. Serum (●) and clot (○) RP 59500 concentration versus time (means  $\pm$  standard errors of the means [bars] for 23 rabbits).

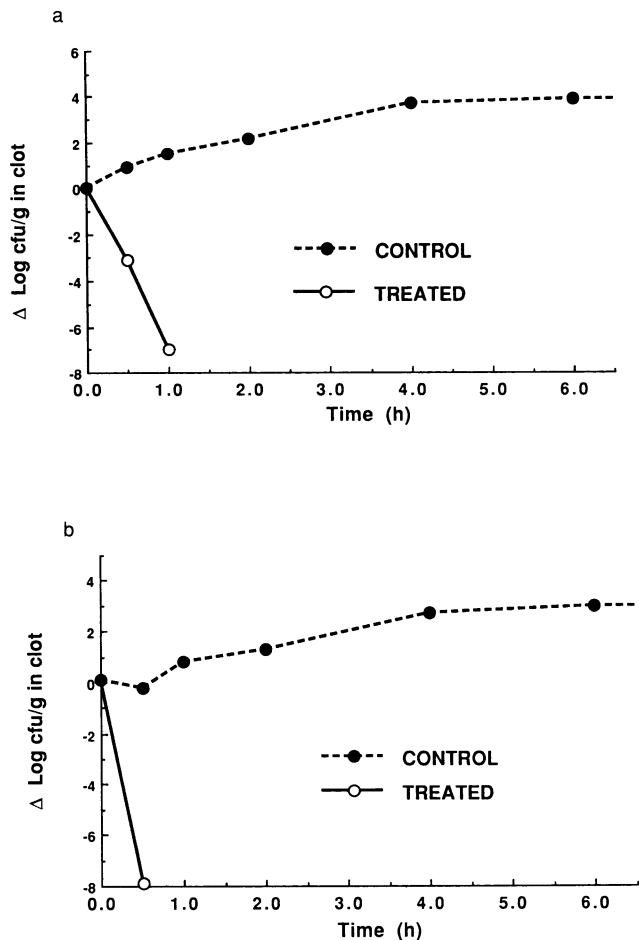


FIG. 2. In vivo killing of methicillin-susceptible (a) and methicillin-resistant (b) *S. aureus* by RP 59500. Δ, mean log difference between number of CFU at baseline and that recovered at the indicated times afterwards.

untreated animals, the colony counts increased by 3.0 and 4.0 log<sub>10</sub>, respectively, in 24 h. After therapy with RP 59500, the clots were sterilized in less than 1 h in both situations.

In the untreated animals infected with methicillin-susceptible (Fig. 3a) or methicillin-resistant (Fig. 3b) *S. epidermidis*, the colony counts increased in each case by 1.5 log<sub>10</sub> over 24 h. After the infusion of RP 59500, there were reductions in counts of 0.5 and 1.5 log<sub>10</sub>, respectively.

With *E. faecalis* (Fig. 4) in treated and untreated animals, the colony counts stayed almost the same during the entire 24 h of the experiment. After treatment, no lowering of the initial inocula was encountered.

**DISCUSSION**

As vancomycin-resistant strains of *Staphylococcus* (15) and ampicillin-resistant (14) and vancomycin-resistant (6) strains of enterococci are emerging, the need to develop new agents like RP 59500 is obvious. As demonstrated in our present study, methicillin-susceptible and -resistant strains of both *S. epidermidis* and *S. aureus* are generally inhibited by concentrations of less than 1 μg/ml (4, 7, 8), while enterococci are inhibited by concentrations of 4 to 8 μg/ml (5). As observed before, raising the inoculum by 100 to 10,000 CFU/ml did not seem to modify the in vitro activity of

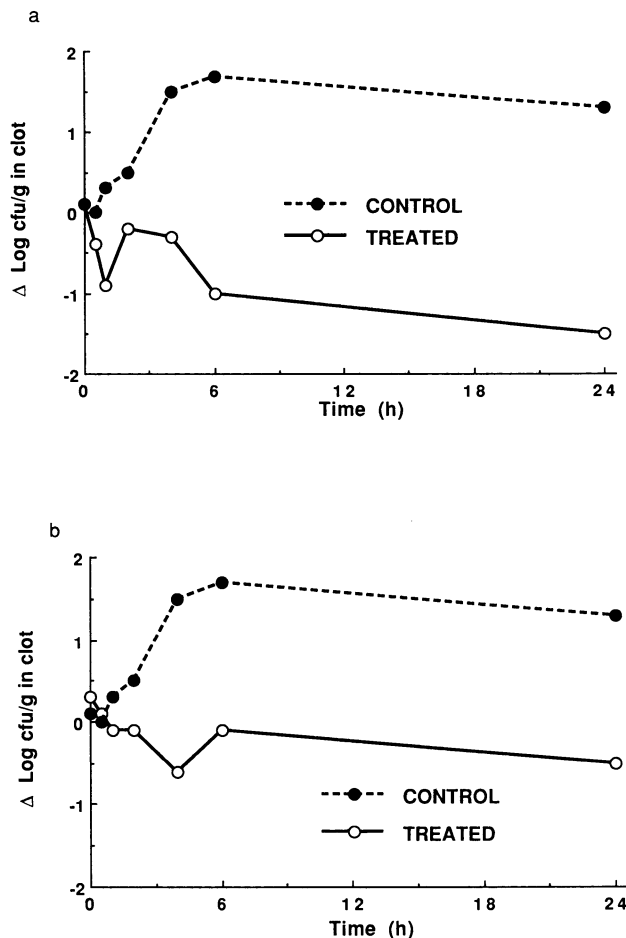


FIG. 3. In vivo killing of methicillin-susceptible (a) and methicillin-resistant (b) *S. epidermidis* by RP 59500. Δ, mean log difference between number of CFU at baseline and that recovered at the indicated times afterwards.

streptogramin against staphylococci (5, 7) but did influence the in vitro activity of the agent against *E. faecalis*, as the MIC increased from 8 to 32 μg/ml. In general, *E. faecalis* is less susceptible than staphylococci to this agent.

The bactericidal activity of this compound has not been thoroughly investigated, but our present data suggest that the MICs and MBCs of RP 59500 for all strains studied are

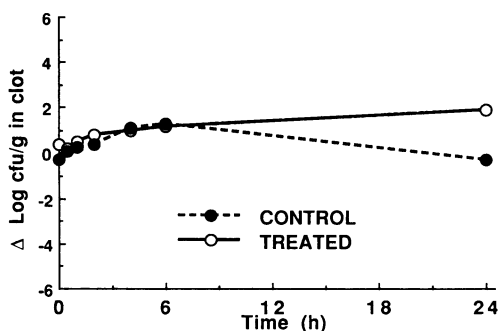


FIG. 4. In vivo killing of *E. faecalis* by RP 59500. Δ, mean log difference between number of CFU at baseline and that recovered at the indicated times afterwards.

TABLE 2. RP 59500 pharmacokinetic parameters<sup>a</sup>

Site of action	AUC <sub>0-t</sub> ( $\mu\text{g} \cdot \text{h/ml}$ )	AUC <sub>0-∞</sub> ( $\mu\text{g} \cdot \text{h/ml}$ )	t <sub>1/2β</sub> (h)
Serum	36.5 ± 2.8	37.1 ± 2.9	0.34 ± 0.01
Clot	12.6 ± 1.0	18.8 ± 1.9	3.8 ± 0.4

<sup>a</sup> Results are measures of dispersion (means ± standard errors of the means for 23 experiments). The ratio of the AUCs for clots to the AUCs for serum is 0.49. AUC<sub>0-t</sub>, AUC from time zero to a given time; AUC<sub>0-∞</sub>, AUC from time zero to infinity; t<sub>1/2β</sub>, half-life at β phase.

similar as long as the inoculum is  $\leq 10^5$  CFU/ml. At a large inoculum of  $10^7$  CFU/ml, the bactericidal activity of this new agent against *S. aureus* was unchanged, but the inoculum size greatly affected its bactericidal activity against *S. epidermidis* and *E. faecalis*. Using a large inoculum of  $10^6$  CFU/ml, Brumfitt and Hamilton-Miller have made similar observations (3). A postantibiotic effect against several pathogens was observed (3). This prolonged growth inhibition may be due to RP 59500's irreversible interaction with the ribosome.

There are limited data on the efficacy of RP 59500 in experimental animals, and its pharmacokinetics in humans is now being investigated. However, the pharmacology of the two main components of pristinamycin, from which RP 59500 is derived, has been evaluated (9). In our experiments, the dose of RP 59500 was chosen to reach levels in serum comparable to those achieved with pristinamycin in humans (9). As expected, the elimination half-life of RP 59500 in serum in rabbits was shorter than that observed with pristinamycin in humans. Our present research demonstrates clearly that the half-life at β phase of this compound is much shorter in serum than in fibrin clots and that the penetration ratio as evaluated by the AUCs in tissue and serum is close to 50%, suggesting that this streptogramin penetrates well into fibrin clots (Table 2). It must be noted, though, that in the use of the AUC from time zero to infinity to calculate this penetration ratio, 33% of the clot AUC is due to extrapolation.

Our model allows the study of the pharmacodynamic interactions between RP 59500 and the bacteria embedded in fibrin clots (2, 10). Because bacteria interact in vivo with a number of different substances that are involved in clot formation and, therefore, in tissue repair, we believe that the fibrin clot is a particularly relevant and useful model. The clumping factor of *S. aureus*, for instance, adheres to fibrinogen, which is converted to the insoluble fibrous protein fibrin during clotting. Dextran produced by certain strains of streptococci can also bind to fibrinogen. Furthermore, in humans, several bacteria can interact with both platelets and fibronectin. For example, *S. aureus* binds to fibronectin, as does the lipoteichoic acid of *Streptococcus pyogenes*. Fibronectin, in addition, binds to fibrinogen and platelets, assisting in their aggregation.

All of these factors contribute to create for the bacteria a protective environment which favors survival of pathogens. Moreover, fibrin clots are extremely hard to penetrate and the amount of antibiotic that reaches the clots varies from one antibiotic to another and is affected by several factors, including routes of administration of antibiotics and protein binding, to name just a few. Even though RP 59500 is 90% bound to protein, the penetration of this compound over several hours was good and the levels observed in the clots were, at least for *S. aureus* and *S. epidermidis*, above the MICs of the agent for these pathogens. *S. aureus* behaved

quite differently from *S. epidermidis*, and complete sterilization of the *S. aureus* clots was observed within the first hours of the experiments. In contrast, although the MICs for *S. epidermidis* were in general 1 dilution lower than the MICs for *S. aureus*, RP 59500 could not sterilize the fibrin clots but could maintain continuous activity throughout the 24-h experiments. This major difference in response can most likely be explained by the limited bactericidal activity of RP 59500 against *S. epidermidis* at a large inoculum. The inoculum used for our in vivo experiment was  $10^7$  to  $10^8$  CFU/g. In our model, RP 59500 had limited in vivo activity against *E. faecalis*, as the concentration reached in clots was lower than the MIC of the antibiotic for this pathogen. In the present experiments, concentrations in plasma did not exceed 100  $\mu\text{g/ml}$  and the saturable protein binding at a concentration of 250  $\mu\text{g/ml}$  that we have observed did not influence penetration or efficacy, as the mean protein binding observed, at a concentration between 25 and 100  $\mu\text{g/ml}$ , was 90%. As we have observed a possible saturable process at concentrations of 250  $\mu\text{g/ml}$ , if higher concentrations are reached in serum, one may expect more free drug to diffuse in tissues, thus improving the potential activity of this new compound.

Although the experiments were not designed to study the postantibiotic effect, the absence of regrowth of *S. epidermidis* even though the levels in fibrin clots were lower than the MICs for the pathogens during at least 12 h may suggest that this agent induces an in vivo postantibiotic effect (5–12). As shown in our previous studies with several β-lactams, we have never observed any postantibiotic effect and regrowth of bacteria always recurred as the antibiotic concentration reached levels below the MIC for the pathogen (10). RP 59500 is a potent new agent which deserves further investigation.

#### ACKNOWLEDGMENT

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