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Efficacy, Toxicity, and Pharmacokinetics of Pentoxifylline and Its Analogs in Experimental *Staphylococcus aureus* Infections

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Pentoxifylline (PTX), a drug that improves neutrophil function in vitro, has been shown to protect neonatal mice against death from experimental staphylococcal infection in vivo at a dose of 50 mg/kg. Using a total of 774 neonatal mice, the effects of various doses of PTX were examined and compared with the effects of three analogs: HWA-448, HWA-285, and A81-3138. A subcutaneous abscess was induced with 10^8 *Staphylococcus aureus*, and drug or saline was given daily subcutaneously from 2 days before to 4 days after infection. Noninfected animals (given saline without *S. aureus*) had 0% mortality (0 of 66), and infected animals without drug (given saline) had a mortality of 70% (161 of 231). PTX and HWA-448 showed the greatest protection among the drugs tested at 15 mg/kg with mortality rates of 27 and 38%, respectively (Kaplan-Meier method, $P = 0.0001$ and 0.0004, respectively). HWA-285 was most protective at 25 mg/kg (mortality, 45%; $P = 0.0046$) and A81-3138 was most protective in animals at 15 mg/kg (mortality, 42%; $P = 0.0045$). PTX, HWA-448, HWA-285, and A81-3138 at doses of 200, 100, 100, and 50 to 75 mg/kg, respectively, were toxic as shown by worsened weight loss and increased mortality in animals when compared with infected animals without drug. PTX and its analogs decrease mortality from experimental infections at lower doses but are toxic at higher doses. Pharmacokinetic characteristics of the drugs were similar except that HWA-285 produced lower concentrations in serum and A81-3138 showed a dose-dependent kinetics (longer half-life at a higher dose).

Pentoxifylline (PTX), a methylxanthine, has been shown to improve in vitro chemotactic responses of rabbit and human neutrophils (PMNs) (9, 15, 19). In animal models, PTX increases accumulation of PMNs into abscesses and sites of inflammation and protects animals from death in experimental infections (7, 19). Although in vivo studies in experimental murine infections reveal protective effects of lower administered dosages of PTX (50 mg/kg), in vitro studies on PMNs show that positive effects require higher doses that may not be achieved in the bloodstream (i.e., concentrations of ≥100 μg/ml). One possibility to explain this seeming discrepancy is that the protective effects of PTX are mediated by its fine-tuning effects on the inflammatory response and its moderating influence on the immunopathologic consequences of infections by suppression of the synthesis and the PMN-priming activity of tumor necrosis factor (TNF) (17, 18). Both drug effects on TNF require low concentrations of PTX. Another possible explanation for this discrepancy is that other methylxanthine analogs or metabolites of PTX are more active than PTX itself and, therefore, are responsible for the protective effects at lower PTX concentrations.

In this study, we compared the protective effects of PTX with those of three analogs that were available to us in experimental infection in neonatal mice. Aside from the hope of finding useful information regarding structure (protective)-function relationships that might provide a clue for the possible presence of a more active metabolite of PTX, such study may result in the discovery of safer and more active compounds. What we found instead were the relatively narrow therapeutic indices of all drugs, which necessitated studies of selected pharmacokinetic characteristics of the drugs.

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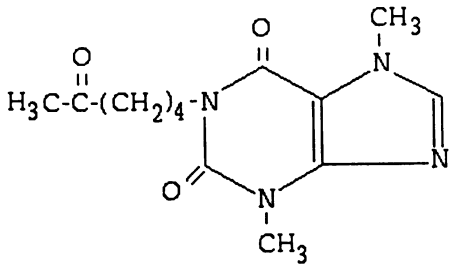
MATERIALS AND METHODS

Drugs. PTX and the three analogs, HWA-448, HWA-285, and A81-3138 (HWA-138), were obtained from Hoechst-Roussel Pharmaceuticals Inc. (Somerville, N.J.). The molecular structures of the compounds are shown in Fig. 1. The chemical names of the compounds are as follows. PTX is 1-(5-oxohexyl)-3,7-dimethylxanthine; HWA-448 is 7-ethoxymethyl-1-(5-hydroxy-5-methylhexyl)-3-methylxanthine; HWA-285 is 3-methyl-1-(5-oxohexyl)-7-propylxanthine; and A81-3138 (or HWA-138) is 1-(5-hydroxy-5-methylhexyl)-3-methylxanthine.

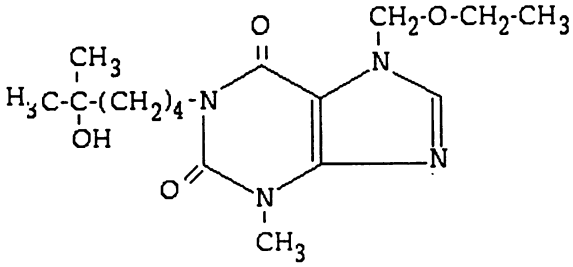
High-pressure liquid chromatographic (HPLC) analysis of the drugs used for injection into the mice demonstrated that PTX, HWA-448, and HWA-285 did not contain detectable amounts of the other drugs, and the respective purities of the drug samples were 98, 93, and 92%. The A81-3138 sample was 85.7% pure, and it contained 1.8% PTX, 5.8% HWA-448, and 6.7% HWA-285.

Antimicrobial activity. To determine whether PTX or its analogs per se exhibit antimicrobial activity, we measured MICs using standard microdilution testing (6). PTX, HWA-448, and HWA-285 were tested in 11 1-to-5 dilutions, at final concentrations ranging from 1.28 to 12.5×10^6 ng/ml, using 5×10^5 CFU of *Staphylococcus aureus* (502A; American Type Culture Collection, Rockville, Md.) in Mueller-Hinton broth per well, in a final volume of 0.2 ml. A81-3138 was similarly tested at concentrations ranging from 0.32 to 3.125×10^6 ng/ml. One set of control wells contained bacteria but no drug, while another contained Mueller-Hinton broth but neither drug nor bacteria. The tests were performed on four separate occasions.

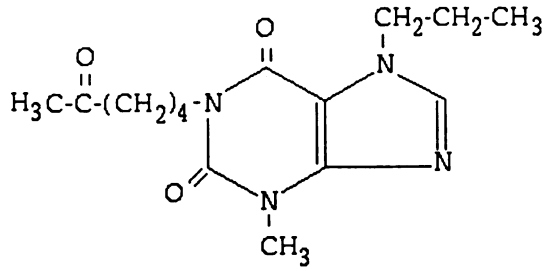
Experimental infection in neonatal mice. Neonatal mice (C57BL/6J; Jackson Laboratory, Bar Harbor, Maine) between the ages of 2 to 4 days were administered subcutaneous (s.c.) injections of 1×10^8 to 2×10^8 CFU of *S. aureus*



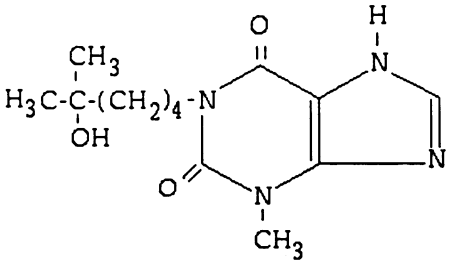
PENTOXIFYLLINE



HWA-448



HWA-285



A81-3138 (HWA-138)

FIG. 1. Molecular structures of PTX, HWA-448, HWA-285, and A81-3138 (or HWA-138).

TABLE 1. Number of neonatal mice used for each group

Dose (mg/kg)	No. of mice				
	Saline (no drug)	PTX	HWA-448	HWA-285	A81-3138
0	231[a]				
0	66				
1		10	9	9	9
5					35
15		26	34	16	33
25		32	44	38	
50		60	13	15	12
75					14
100		26	14	14	
200		14			

[a] These mice were not infected with *S. aureus*, whereas all the other mice were infected.

(502A). Body weights and mortality were determined daily for 4 days thereafter.

To determine the protective effects of PTX or analog, we administered drug or saline s.c. (at the side opposite the bacterial injection site) every 24 h for 6 days beginning 48 h before *S. aureus* challenge up to 96 h after *S. aureus* challenge. To minimize the possible effects of litter variability, mice from each litter were not assigned to one group but were distributed at random among the control and various treatment groups. The six groups were as follows: noninfected controls receiving s.c. injection of saline without *S. aureus*, infected nontreated controls receiving s.c. injection of *S. aureus* and saline without drug (neither PTX nor analog), and four infected treatment groups receiving *S. aureus* and pentoxifylline, HWA-448, HWA-285, or A81-3138. Table 1 summarizes the number of animals used in each group.

Statistical and pharmacokinetic analyses. In comparing weights between treatment groups, we used the daily weight index. The weight index for the group was obtained by summing the weights of the living mice each day and dividing by the total number of mice in the group when treatment started. Thus, the weight index is the average weight of the mice in each treatment group, in which dead mice are counted but each given a weight of zero. This method avoids the misgrading of values that would occur if dead mice were not accounted for or if dead mice were assigned their weights at death. In the first instance, dead mice would receive weight values equal to the average weight of the surviving mice in the group; in the second instance, dead mice would be given weight values higher than those of surviving mice that continued to lose weight.

Survival curves were analyzed by the Kaplan-Meier product-limit method. Tests of significance of the differences in the survival curves of infected mice in the control group (infected mice without drug treatment) versus infected mice in each of the drug treatment groups were performed by using the log-rank test. The calculations were performed by using the SAS statistical package (13).

The pharmacokinetics of PTX and its analogs were analyzed by using an open two-compartment model (12). The elimination phase of all the drugs studied followed first-order kinetics: $\text{drug}_t = \text{drug}_0 \times e^{-kt}$, where drug_t is the drug concentration at time interval t, drug_0 is the drug concentration at the beginning of the time interval, and k is the kinetic constant. The value of the kinetic constant k was calculated as the negative slope of the natural logarithm of the above equation by using regression analysis (13): $\ln \text{drug}_t = \ln \text{drug}$

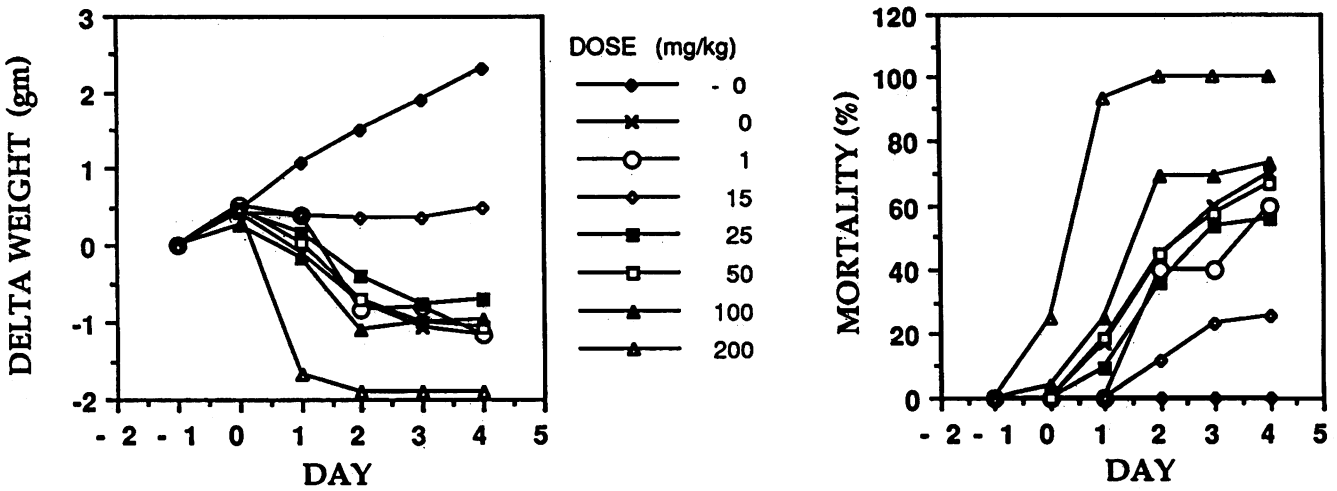


FIG. 2. Cumulative percent mortality (right panel) and change in weight index (left panel) for day −1 to day 4 for PTX doses ranging from 0 to 200 mg/kg. The top curve, labeled −0, is for noninfected, nontreated (saline-treated) neonatal mice. All other curves are for neonatal mice that were infected with *S. aureus* and treated with the doses indicated.

− *kt*. The earliest blood samples were obtained 0.5 h after administration of the drugs. We report the drug concentrations at 0.5 h, although the true peak drug concentration may occur at a slightly shorter time interval.

Quantitative analyses of PTX and analogs by HPLC. The method used for quantitative drug analysis was that described in detail previously (3). The HPLC system consisted of a model 510 pump (Waters Associates, Inc., Milford, Mass.), a model C6W injector (20-μl sample loop) (Valco Instruments Co., Houston, Tex.), and a model SM4000 programmable wavelength detector and a model CI-10B peak integrator (LDC/Milton Roy, Riviera Beach, Fla.). The system utilized a C-18 reversed-phase column (3.9 by 300 mm) with 10-μm particle size (Waters Associates). Degassing of the mobile phase was accomplished by aspiration each day before analysis.

After s.c. injection of either PTX or analog (HWA-448, HWA-285, or A81-3138), 70 μl of whole blood was collected from each neonatal mouse and placed immediately into 4 ml of acetonitrile. The samples were mixed and centrifuged at 3,000 × *g* for 10 min. A sample of precisely 3.5 ml of the supernatant was removed, and an internal standard, 3-isobutyl-1-methylxanthine (10 μl of a 200-μg/ml solution) was added to each sample. The sample was mixed and evaporated to dryness at 47°C under a stream of nitrogen. Hexane (1 ml) was added to the residue in each tube and mixed for 30 s, after which 0.5 ml of HPLC-grade water was added. The contents of the tubes were mixed and centrifuged at 3,000 × *g* for 10 min, and a 200-μl sample of the aqueous phase was removed for chromatographic analysis. A81-3138, PTX, isobutylmethylxanthine, HWA-448, and HWA-285 exhibited retention times of 8.4, 10.5, 11.5, 27.3, and 33.0 min, respectively, using a mobile phase of 24:76 acetonitrile to water and a flow rate of 1 ml/min. Since the animals were exsanguinated for these studies, they were used specifically for this study.

RESULTS

Microbiologic effects of PTX and analogs. PTX and all three analogs had no antimicrobial activity against *S. aureus* up to concentrations of 12.5 mg/ml for PTX, HWA-448, and HWA-285 and up to 3.125 mg/ml for A81-3138.

Effect of PTX and analogs on weight loss. In this study, we calculated a daily weight index for each treatment group by summing the weights of the living mice each day and dividing by the total number of mice in the group when treatment started. Figures 2 to 5 illustrate the change in the weight index for each of the treatments from days −1 to 4. Following infection, there was a steady drop in the value of the weight index in the nontreated group. Using the weight index as the endpoint, the optimal protective dose was 15 mg/kg for PTX, 15 to 25 mg/kg for HWA-448, 1 to 25 mg/kg for HWA-285, and 15 mg/kg for A81-3138. All drugs were toxic at the highest doses used since decreases in weight index at these doses were greater compared with the nontreated infected controls. Among the drugs used, toxic potential was greatest with A81-3138 since toxicity was evident at the 50-mg/kg dose.

Effect of PTX and analogs on mortality. Noninfected animals (given saline without *S. aureus*) had 0% mortality (0 of 66), and infected animals without drug had a mortality of 70% (161 of 231) by day 4. As shown in Fig. 2 and 3, PTX and HWA-448 showed the greatest protection among the drugs tested at 15 mg/kg, with mortality rates of 27 and 38%, respectively. HWA-285 was most protective at 25 mg/kg (mortality, 45%) and A81-3138 at 15 mg/kg (mortality, 42%) (Fig. 4 and 5). Using these optimum protective dosages, survival curves were significant when compared with those in the nontreated infected controls, as shown by the Kaplan-Meier product-limit method (Fig. 6). The *P* values for PTX, HWA-448, HWA-285, and A81-3138 were 0.0001, 0.0004, 0.0046, and 0.0045, respectively. PTX, HWA-448, HWA-285, and A81-3138 at doses of 200, 100, 100, and 50 to 75 mg/kg, respectively, were toxic, as shown by increased mortality when compared with infected animals without drug.

Concentrations of PTX and analogs in sera. To measure the kinetic constants for elimination (*k*) and the half-lives of the drugs, concentrations in sera of neonatal mice after subcutaneous injection were quantitated by HPLC at various time intervals after drug administration. Since blood collection necessitated exsanguination, a separate group of neonatal mice was used specifically for this study, and each mouse contributed a single data point. With two notable exceptions,

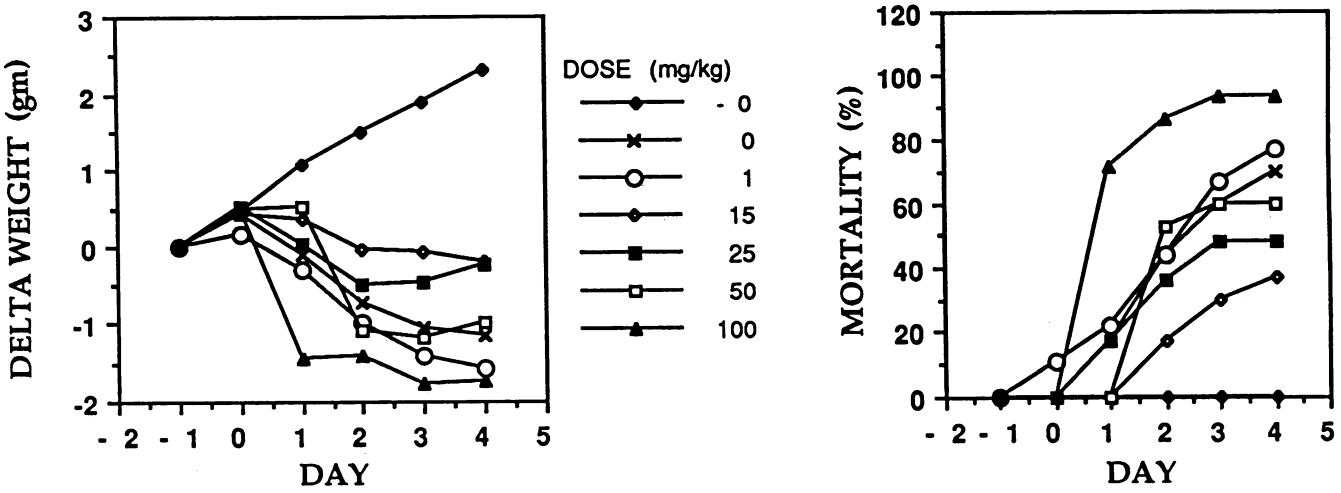


FIG. 3. Cumulative percent mortality (right panel) and change in weight index (left panel) for day −1 to day 4 for HWA-448 doses ranging from 0 to 100 mg/kg. The top curve, labeled −0, is for noninfected, nontreated (saline-treated) neonatal mice. All other curves are for neonatal mice that were infected with *S. aureus* and treated with the doses indicated.

the kinetic constants and half-lives of PTX and the three analogs were all very similar. The concentrations achieved at 0.5 h for a given dose approximated each other for PTX, HWA-448, and A81-3138, but that for HWA-285 was considerably lower. The results are summarized in Fig. 7 and Table 2. At the 5-mg/kg dose, PTX appeared to be more rapidly eliminated, but only a few data points are available for calculation because the drug was not detectable after the 1-h sample; consequently, no curve was drawn on the figure. This tentative conclusion needs to be confirmed.

With regards to A81-3138, there is definite dose-response kinetics as shown by a slower decline in concentration in serum with time observed at a dose of 50 mg/kg compared with lower doses. At 7 h after a 50-mg/kg dose, A81-3138 concentration still exceeded 10 μg/ml, whereas for PTX and other analogs at similar doses, the concentrations in serum were ≤1 μg/ml. This characteristic may contribute to the toxicity of A81-3138.

DISCUSSION

Xanthine derivatives have previously been shown to affect host defense mechanisms, particularly PMN function. For example, isobutylmethylxanthine improved the in vitro chemotactic response of hypofunctional human PMNs (2). On the other hand, 7-β-hydroxybenzylopiperazinopropyl theophylline inhibited spontaneous migration and reduced leukocyte responses to various types of stimulation (11). More recently, PTX was shown to exert various effects on the PMN and the inflammatory process. Originally, it was shown to improve the chemotactic response of normal rabbit peritoneal PMNs to the synthetic bacterial chemotactic factor *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine beginning at a concentration of 14 μg/ml (15). This improved response was associated with a rise in PMN phosphoinositide levels. It was therefore concluded that the improvement in function was related to membrane skeletal stabilization mediated through a drug effect on phosphoinositides. Similar improvement in chemotaxis with PTX has been subsequently reported in normal and dysfunctional human PMNs (9, 19), but concentrations needed for maximum improvement of function were between 1 and 10 mM (278 and 2,780 μg/ml), which in this study include levels in the toxic range. In one study, improvement of chemotactic response was noted when mononuclear cells were present in the mixture

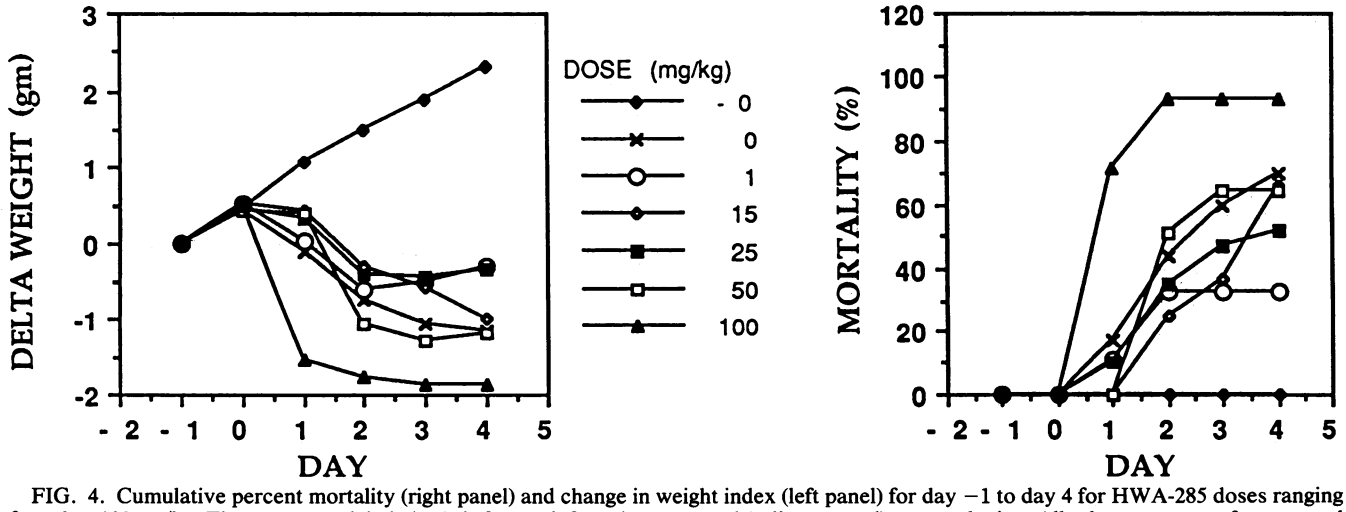


FIG. 4. Cumulative percent mortality (right panel) and change in weight index (left panel) for day −1 to day 4 for HWA-285 doses ranging from 0 to 100 mg/kg. The top curve, labeled −0, is for noninfected, nontreated (saline-treated) neonatal mice. All other curves are for neonatal mice that were infected with *S. aureus* and treated with the doses indicated.

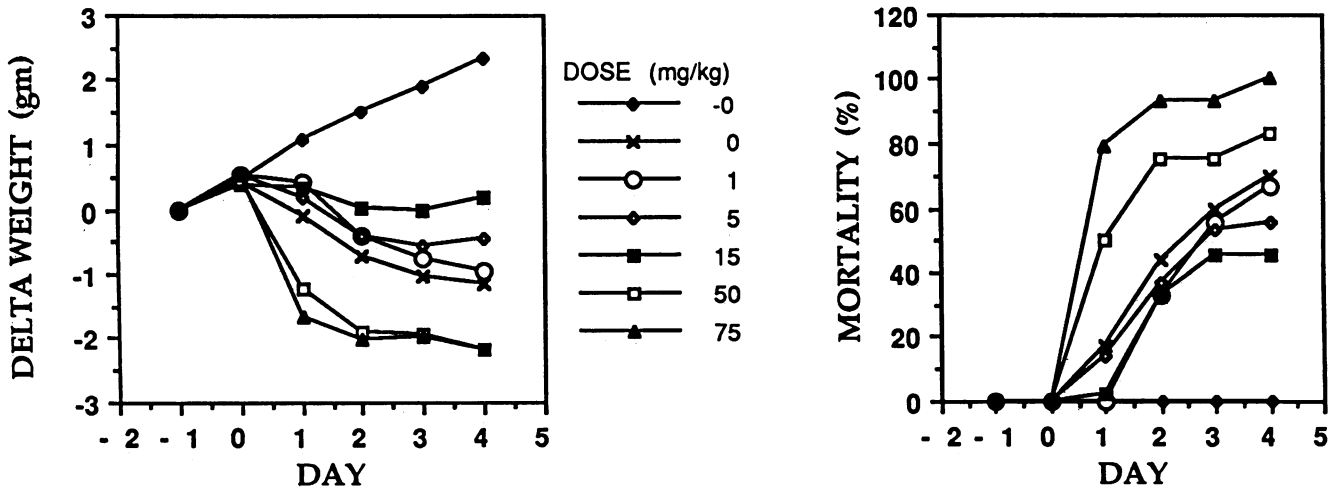


FIG. 5. Cumulative percent mortality (right panel) and change in weight index (left panel) for day −1 to day 4 for A81-3138 doses ranging from 0 to 75 mg/kg. The top curve, labeled −0, is for noninfected, nontreated (saline-treated) neonatal mice. All other curves are for neonatal mice that were infected with *S. aureus* and treated with the doses indicated.

(but not when they were absent) or by treating PMNs with TNF-α or supernatants from endotoxin-stimulated mononuclear cells. These findings suggest that the seemingly improved response to chemotactic attractant with PTX is due to its suppressive effects on the priming of PMNs by TNF released by mononuclear cells. Thus, PTX may limit the potential for preactivated or primed PMNs to subsequent deactivation (5). Other PMN responses that appear to be reduced or regulated by PTX at high concentrations include adherence, aggregation, and cell swelling (8, 18), degranulation (18), and superoxide production (1, 18). Note that in these studies (9, 15, 19), the effect of PTX was biphasic, with enhancement of PMN function at low dose and impairment in PMN responses at high dose, suggesting that PTX is toxic in higher doses.

An important effect of PTX which may be critical for host response to infection is its ability to increase cell membrane fluidity and cell deformability, not only of erythrocytes but also of leukocytes, including the PMNs (10, 14). There is evidence that PTX also affects host defense via nonmembrane mechanisms. Strieter and co-workers (17) reported that PTX at a concentration of 10^{-5} M (2.8 μg/ml) inhibited mRNA expression and production of TNF-α by lipopolysaccharide-stimulated mononuclear phagocytes. Other meth-

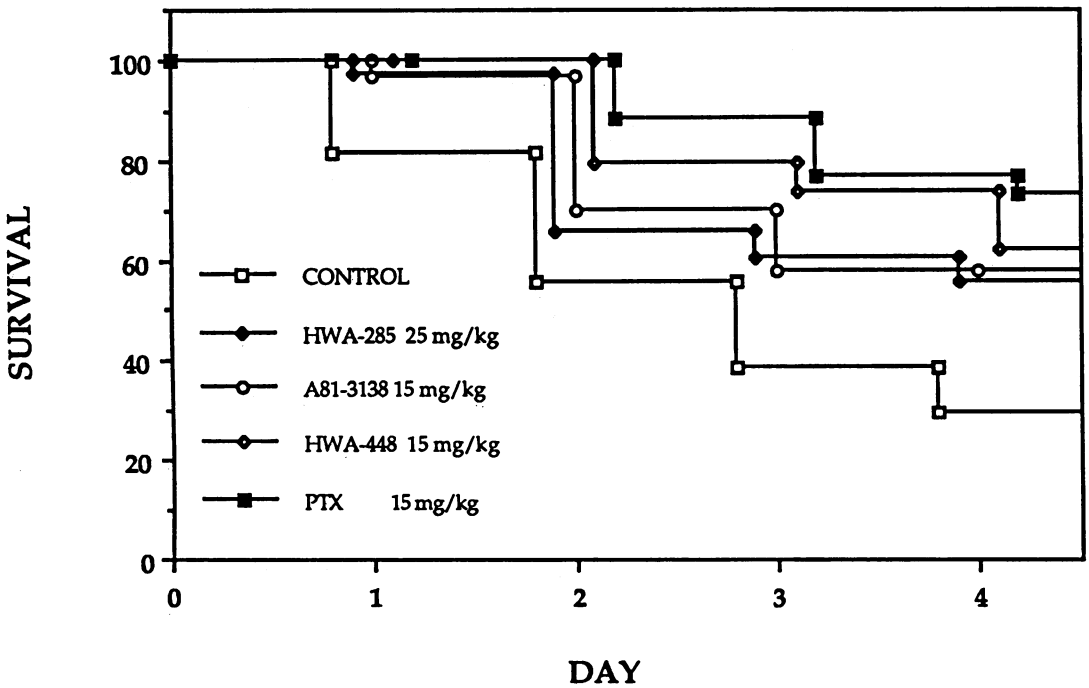


FIG. 6. Survival curves for neonatal *S. aureus*-infected mice. The control mice received saline without drug, whereas the other mice were treated with optimum doses of the drugs as indicated. All data points should fall exactly on days indicated, but minor adjustments were made to avoid overlapping of the points.

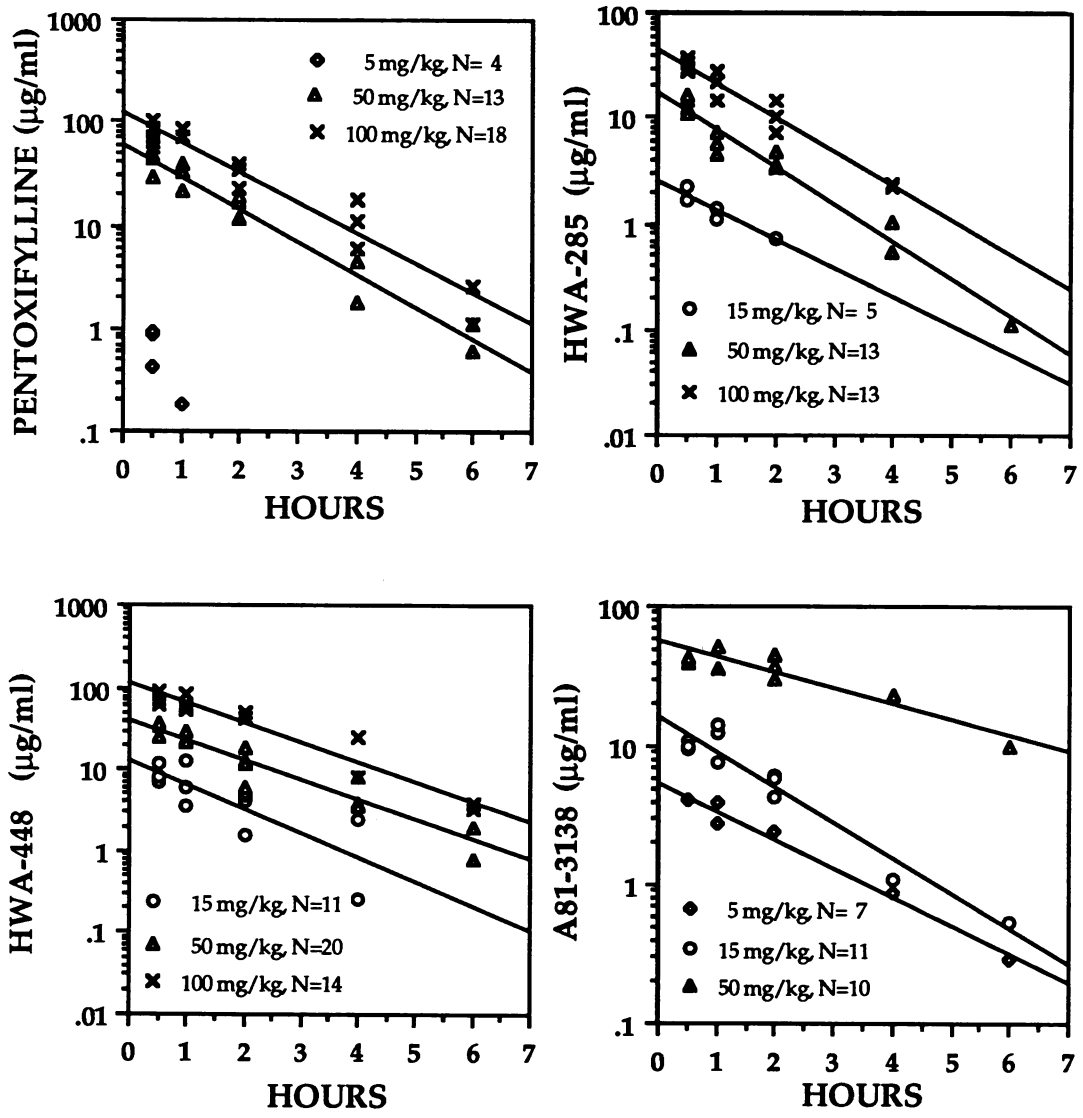


FIG. 7. Semilog plots of the concentrations in blood PTX, HWA-285, HWA-448, and A81-3138 as a function of time in hours after drug administration. These are composite curves in which each data point was obtained with the blood from one mouse, and *N* is the number of mice used to obtain each curve. The slopes of the lines were used to estimate the kinetic constants.

ylxanthines and dibutyryl cyclic AMP produced the same inhibitory effects, suggesting that the suppressive effect of the drug is mediated through intracellular cyclic AMP and confirming what others had reported earlier, that PTX inhibits phosphodiesterase and elevates cyclic AMP levels in PMNs and other cells (1, 4, 16). In addition to the direct effect of PTX on TNF-α synthesis, there is evidence, as noted above, that at relatively low concentrations (beginning at 0.1 μg/ml), it may also block the priming effect of preformed TNF-α on PMNs (18), thus minimizing the potential to PMN dysfunction by deactivation (5). Although PTX seems to have multiple regulatory activities on the PMN, many of these can be explained by its effects on the few cellular mechanisms discussed above. For example, the effects of PTX on membranes may explain its effects on cell deformability, inhibition of PMN priming by TNF-α, diminished adherence and aggregation, reduced superoxide production, and improved locomotory responses. Whether the inhibition of TNF synthesis can be explained by the membrane effects of PTX is not clear at this time. The fact that mRNA expression of TNF is abrogated suggests that the site of action is extramembranous. Regardless of the more basic mechanisms involved, the moderating effects of PTX on TNF production and the tissue-damaging tendencies of preactivated PMNs and its positive rheologic effects combine to provide ample explanation of its protective activity in infected animals. Beyond doses that produce a fine-tuning effect on the inflammatory response, PTX or analog becomes toxic, reflecting the importance of maintaining host defenses at a certain level of activity. This is reminiscent of the adverse effects of most anti-inflammatory agents.

In summary, PTX protected neonatal mice against weight loss and death from experimental infections and had greater activity than the analogs tested. The results also showed that, similar to other drugs with anti-inflammatory effects, PTX and analogs have a narrow therapeutic range since toxicity is evident at higher but achievable concentrations in serum. Finally, the results suggest that TNF- and PMN-induced tissue damage, which are suppressed by low drug concentrations similar to the protective concentrations

TABLE 2. Pharmacokinetic characteristics of s.c. administered PTX and analogs

Drug	Dose (mg/kg)	Concn at 0.5 h (mg/ml)	k (h^{-1})	$t_{1/2}$ (h)
PTX	5	0.74	2.7[a]	0.3[a]
	50	39.8	0.72	1.0
	100	74.4	0.67	1.0
HWA-448	15	9.01	0.69	1.0
	50	32.1	0.56	1.2
	100	78.2	0.55	1.3
HWA-285	15	1.87	0.62	1.1
	50	13.38	0.82	0.8
	100	31.69	0.74	0.9
A81-3138	5	4.16	0.48	1.5
	15	10.1	0.59	1.2
	50	41.8	0.26	2.7

[a] These values are probably inaccurate (calculated from only three points) because many samples had PTX concentrations that were beyond the detection limits of our assay.

shown in this study, are important cofactors that have an influence on morbidity and mortality in serious bacterial infections.

ACKNOWLEDGMENTS

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