MIC VALUES DO NOT PREDICT THE INTRAPHAGOCYTIC KILLING OF *STAPHYLOCOCCUS AUREUS* BY NAPHTHALENIC ANSAMYCINS

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Ten naphthalenic ansamycins were compared for their ability to kill extracellular or phagocytosed *Staphylococcus aureus* 502A. These included rifamycins, streptovaricins and tolypomycin Y. Although the compounds differed markedly in killing extracellular *S. aureus*, there was surprisingly little difference between them in assisting human leukocytes to kill phagocytosed *S. aureus*. In fact, when compared to rifampin, some ansamycins that were less effective in killing extracellular bacteria were more effective in killing phagocytosed bacteria.

These data, together with an analysis of structure and activity, suggested that a specific transport mechanism might be involved. First considered was a vitamin K transport mechanism. Indeed warfarin, a vitamin K antagonist, blocked the ability of rifampin to kill phagocytosed *S. aureus*, as did the coumarins, novobiocin and coumarin-3-carboxylic acid. However, direct evidence for a vitamin K transport mechanism could not be obtained using vitamin K preparations.

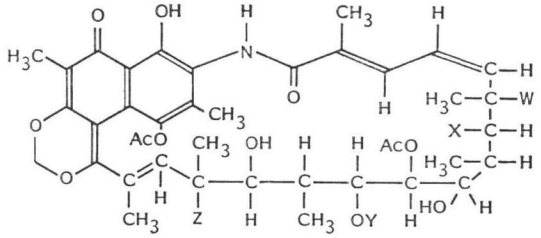
The fused phenolic, bicyclic system common to all of these ansamycins was tentatively considered to be the portion necessary for phagocyte penetration.

ROUS and JONES[1] wrote in 1916 that "Living phagocytes are able to protect ingested organisms from the action of destructive forces in the surrounding fluid. . .". Although they were referring primarily to natural host defense mechanisms, it has since been noted that several pathogenic microorganisms can be similarly protected from the effects of therapeutically applied antimicrobial agents. It is appropriate, therefore, to attempt to understand the transport mechanisms underlying the ability of those few antimicrobial agents that are able to penetrate phagocytes sheltering microorganisms. One rational for studying these mechanisms involves using compounds that resemble an antibiotic already known to penetrate phagocytes. If structure activity relationships emerge, then some knowledge can be gained concerning the transport mechanism.

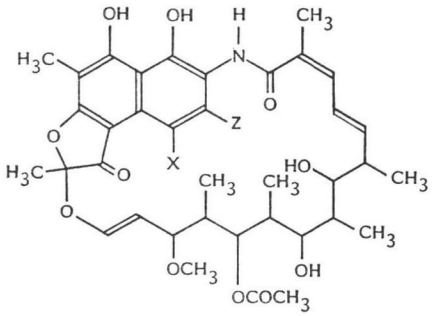
Perhaps the best studies of those antimicrobial agents capable of rapid phagocyte penetration is rifampin (also known as rifampicin). Rifampin's intraphagocytic killing powers were described by LOBO and MANDELL[2], MANDELL[3], and MANDELL and VEST[4]. These results were confirmed and extended by SOLBERG and HELLUM[5], EASMON[6] and by PROKESCH and HAND[7].

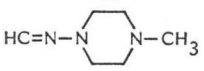
We have studied ten naphthalenic ansamycins[8,9], including rifampin, for their ability to kill phagocytosed *Staphylococcus aureus* 502A[10,11,12]. Quite unexpectedly, all were very effective in killing phagocytosed *S. aureus*. These results were unanticipated because the ten compounds differ markedly in their ability to kill extracellular *S. aureus*. These data, in conjunction with an analysis of structure (Fig. 1) and activity suggest that a specific transport mechanism may be involved in the penetration of phagocytes by naphthalenic ansamycins.

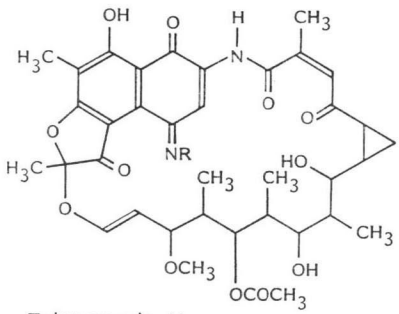
Fig. 1. The chemical structures of naphthalenic ansamycins (streptovaricins, rifamycins, tolypomycin Y), vitamin K_1, warfarin, novobiocin and coumarin-3-carboxylic acid.



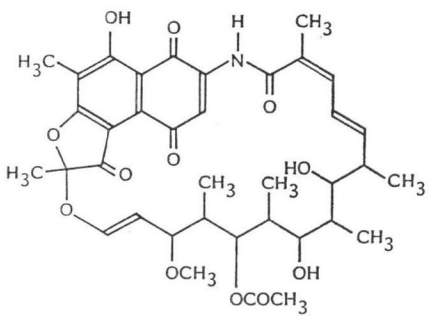
	W	X	Y	Z
Streptovaricin A	OH	OH	Ac	OH
Streptovaricin B	H	OH	Ac	OH
Streptovaricin C	H	OH	H	OH
Streptovaricin D	H	OH	H	H
Streptovaricin G	OH	OH	H	OH



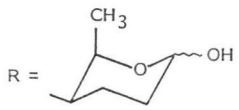
	X	Z
Rifamycin B	OCH_2COOH	H
Rifamycin SV	OH	H
Rifampin	OH	

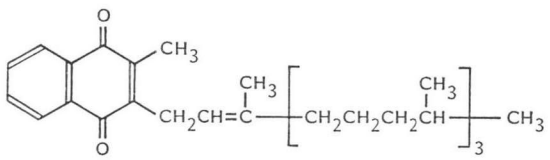


Tolypomycin Y

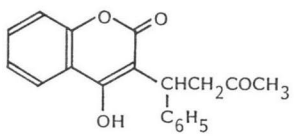


Rifamycin S

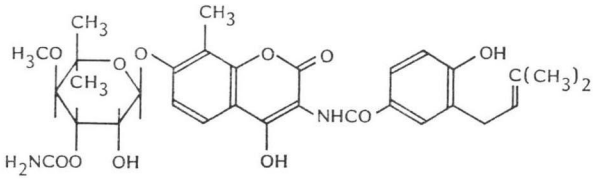




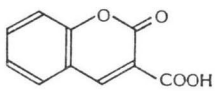
Vitamin K_1



Warfarin



Novobiocin



Coumarin-3-carboxylic acid

Materials and Methods

Growth of *S. aureus* 502A (UC 9116)

S. aureus 502A obtained from the laboratory of G. L. MANDELL, was transferred from Trypticase soy (TS) agar into TYG broth which contains Tryptone, yeast extract and glucose added, respectively, at 5 g, 3 g and 20 g per 1 liter of deionized H_2O. The organism was incubated aerobically at 37°C for 24 hours. The resulting stationary phase culture was washed twice by alternate centrifugation at $5,000 \times g$ and was resuspended into normal saline. Under these conditions of growth, the cell titer at harvest was *ca.* 10^9 bacteria per ml. The organism was diluted to 10^8 cells per ml using Hanks balanced salts solution lacking Ca, Mg and phenol red (HBSS) buffered to pH 7.4 with *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) at 5 mg per ml.

Preparation of Human Leukocytes

Leukocytes were collected from sodium citrate-treated blood after dextran sedimentation. The leukocytes were washed by centrifugation in HBSS/HEPES and diluted to 1×10^7 leukocytes/ml in HBSS/HEPES containing 20% autologous serum.

Assay Protocol

Phagocytosis of *S. aureus* was accomplished by incubating 1 ml of the leukocyte suspension with 1.3×10^8 washed *S. aureus* cells in 1.3 ml of HBSS/HEPES. The resulting suspension was mixed slowly by rocking for 30 minutes at 37°C in a sterile NUNC Cryotubes 90×12.5. Following incubation, a microscopic examination ensured that phagocytosis had occurred. In order to destroy any remaining extracellular bacteria, 60 units of lysostaphin (Schwarz-Mann) were added to the 3 ml preparation[13,14]. The resulting preparation was mixed as before for 30 minutes at 37°C. Following this manipulation the antibiotics, usually dissolved first in dimethyl sulfoxide (DMSO), were added at the appropriate concentration and were incubated in the system for the indicated time period at 37°C. The final concentration of DMSO was $\leq 0.1\%$. DMSO was used for the sake of convenience to decrease the time required for the preparation of the naphthalenic ansamycin solutions. However, similar results were obtained in the absence of DMSO using more dilute stock solutions and allowing a longer time for solubilization in aqueous media (see Table 1). In some experiments, after treatment with lysostaphin, warfarin, novobiocin or coumarin-3-carboxylic acid were added at incremental concentrations ranging between 10 and 1,000 μg per ml. In these experiments it was necessary to select for a physiological variant of *S. aureus* 502A obtained by growing the organism in TYG broth overnight. This seed culture was then used as the final inoculum for the growth procedure described earlier in this section. The variant tested as an extracellular organism was equally as sensitive to rifampin as wild type, but was slightly more resistant to rifampin when tested intraphagocytically.

Warfarin, novobiocin and coumarin-3-carboxylic acid were employed as their sodium salts and were added 10 minutes prior to the addition of antibiotic. Following incubation, 90 mg of trypsin (Difco) were added to the mixture and incubated at 37°C for 15 minutes to destroy residual lysostaphin. The final volumes of all of the mixtures were adjusted to 3.0 ml with normal saline. To remove extracellular antibiotics, the mixtures were washed twice by alternate centrifugation at $5,000 \times g$ followed by resuspension into normal saline and the samples were frozen. After thawing, the mixtures were subjected to sonic disruption for 5 seconds in the cold using a Branson Sonic Oscillator at a power setting of 1. The exposure was sufficient to lyse the phagocytes and to disrupt clumps of *S. aureus* without killing bacteria.

In order to quantify the surviving bacteria, the mixture was diluted at ten-fold increments in normal saline. Two tenth ml volumes of each dilution were plated onto TS agar and were incubated for 24 hours at 37°C. Following incubation, plate counts were made on at least 2 dilutions.

Results

The Use of DMSO in the Solubilization of Naphthalenic Ansamycins

DMSO was used routinely to more quickly dissolve the relatively water insoluble naphthalenic

Table 1. Comparison of naphthalenic ansamycins solubilized in the presence and absence of DMSO on the killing of phagocytosed *S. aureus*.

Naphthalenic ansamycin (10 μg/ml)	cfu *S. aureus* × 10^3 after 90-minute exposure	
	No DMSO	DMSO
None	181	203
Rifampin	4	27
Rifamycin B	5	12
Rifamycin S	7	5
Streptovaricin A	22	28
Streptovaricin C	49	35
Streptovaricin D	39	52

Table 2. Activity of selected ansamycin antibiotics vs. extraphagocytic *S. aureus* 502A.

Antibiotic	MIC (μg/ml)
Rifampin	≤0.02
Streptovaricin A	2.5
Streptovaricin B	2.5
Streptovaricin C	1.25
Streptovaricin D	1.25
Streptovaricin G	0.625
Rifamycin B	1.25
Rifamycin S	0.16
Rifamycin SV	0.16
Tolypomycin Y	1.25

ansamycins. To test the possibility that DMSO was in some way enhancing the penetration of the naphthalenic ansamycins into phagocytes, the antibiotics dissolved with the aid of DMSO were compared to those dissolved initially in aqueous solution. In the latter case more dilute antibiotic stock solutions were employed. As the data in Table 1 show, there were not substantial differences in the activities of the antibiotics prepared by the different methods. These data also indicate that DMSO, at the concentration employed, did not influence the killing of the phagocytosed staphylococci. Therefore, for convenience, DMSO was used to initially dissolve the antibiotics by the procedure described in Methods.

MIC Values for Naphthalenic Ansamycins

The MIC values of naphthalenic ansamycins against *S. aureus* 502A are shown in Table 2. All possessed anti-*S. aureus* activity. Rifampin was clearly the most potent followed by rifamycins S and SV. Streptovaricin G was the only streptovaricin with an MIC value less than 1 μg/ml. Rifamycin B, tolypomycin Y, as well as the streptovaricins A, B, C and D had MIC values greater than 1 μg/ml. Rifampin was 60 to 120 times more potent than the latter group of compounds.

Evidence that the Effects of Naphthalenic Ansamycins were Intracellular

Evidence for intracellular effects of rifampin and the other naphthalenic ansamycins was two-fold. First ampicillin and novobiocin, which have MIC values in the same concentration range as rifampin (≤0.1 μg/ml) vs. *S. aureus* 502A, displayed little activity vs. phagocytosed *S. aureus* 502A. However, in 11 experiments 10 μg of rifampin per ml reduced the number of *S. aureus* by an average of 5-fold during a 90-minute incubation with 50% of the bacteria killed during the first 30 minutes. In contrast, under the same conditions, ampicillin reduced the population only 1.4 fold in 90 minutes and required 180 minutes to kill 50% of the phagocytosed *S. aureus*. Furthermore, novobiocin had no activity vs. phagocytosed *S. aureus* 502A when tested at 1 mg per ml.

Secondly, as Table 3 shows, it could be reasoned that there was an interaction between rifampin and lysostaphin. Lysostaphin was shown recently to kill phagocytosed bacteria[15]. At the concentration of lysostaphin employed in these experiments (*ca.* 20 units per ml) lysostaphin reduced the colony forming units (cfu's) from >1,000 × 10^3 to 220 × 10^3 per ml. Rifampin in the absence of lysostaphin reduced the cfu's to 52 × 10^3 per ml. However, in combination with lysostaphin, rifampin caused a further reduction to 19 × 10^3 cfu's. These results are similar to those reported by CRAVEN and ANDER-

Table 3. The interaction between rifampin and lysostaphin in killing intraphagocytic *S. aureus*.

Lysostaphin (units/ml)	cfu *S. aureus* $\times 10^3$	
	No rifampin	Rifampin (10 μg/ml)
0	>1,000*	52
3	670	37
6	360	29
10	290	23
24	220	19
64	150	14

* The high bacterial count reflects extracellular staphylococci.

Table 4. The killing of phagocytosed *S. aureus* 502A by naphthalenic ansamycins at a concentration of 5 μM in the absence of lysostaphin.

Antibiotic	cfu $\times 10^5$ following 60-minute antibiotic exposure
No addition	125
Rifampin	27
Rifamycin SV	10
Streptovaricin A	28
Streptovaricin C	28
Streptovaricin D	26

SON[16] in 1980.

These data indicate that the effect of lysostaphin at *ca.* 20 units per ml was enhanced greatly by the addition of rifampin. As it was necessary to destroy extracellular staphylococci in these experiments, it is important that the effect of rifampin was substantially greater than the effect of lysostaphin alone. Thus when control assays containing lysostaphin alone are performed in conjunction with those containing rifampin and lysostaphin, it appears valid to employ lysostaphin for the stated reasons.

To further insure that naphthalenic ansamycins kill phagocytosed *S. aureus* in the absence of lysostaphin, the experiment reported in Table 4 was performed. Here four of the antibiotics in addition to rifampin were shown to reduce a population of phagocytosed staphylococci between 5 and 12-fold. The degree of killing by the ansamycins tested was proportional to their effects in the presence of lysostaphin. Again, it appears reasonable to employ lysostaphin for the destruction of extracellular staphylococci.

Comparison of the Rates of Killing of Phagocytosed *S. aureus* by Rifampin and Streptovaricins

Since rifampin has been thoroughly investigated for its ability to kill phagocytosed *S. aureus*, it was employed in these experiments as a paired reference standard. The first criterion used to compare the compounds was the rate of killing, *i.e.*, the time required to kill 50% of the phagocytosed *S. aureus* at a concentration of 10 μg per ml. The approximate times required for 50% destruction in paired experiments were rifampin=20 minutes and streptovaricin A=35 minutes; rifampin=30 minutes and streptovaricin B=30 minutes; rifampin=20 minutes and streptovaricin C=35 minutes; rifampin=20 minutes and streptovaricin D=40 minutes; rifampin=30 minutes and streptovaricin G=25 minutes. The data from the last two paired experiments were selected for presentation (Fig. 2).

When a second criterion of comparison was used, *viz.*, the number of viable bacteria surviving after 80 minutes of incubation, there was little difference between rifampin and streptovaricins A, B, C, D and G. Thus, using two criteria, there was little difference between rifampin and streptovaricins in the rates of killing phagocytosed *S. aureus*.

Comparison in the Rates of Killing of Phagocytosed *S. aureus* by Rifampin, Rifamycins, and Tolypomycin Y

The results with these antibiotics were segregated from those of the streptovaricins because the rifamycins and tolypomycin Y are more closely related to rifampin than are the streptovaricins in terms of their ring structures. In paired experiments at 10 μg/ml the approximate times required for 50%

Fig. 2. The rate of killing phagocytosed *S. aureus* by streptovaricins D and G compared with rifampin.

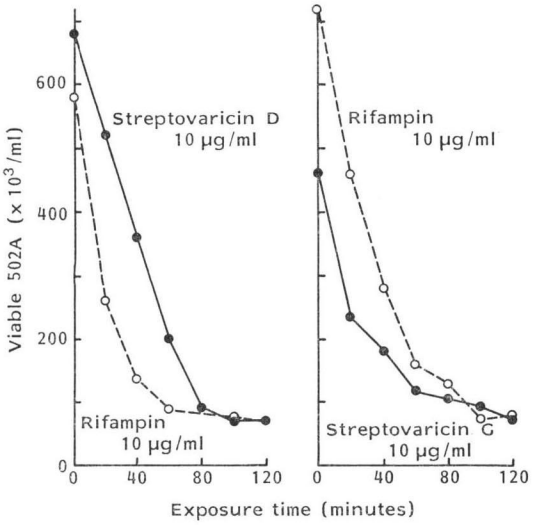
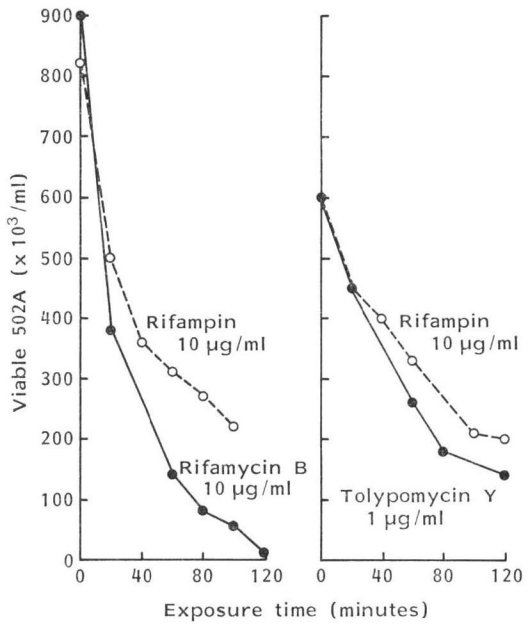


Fig. 3. The rate of killing phagocytosed *S. aureus* by rifamycin B and tolypomycin Y compared with rifampin.



killing of phagocytosed *S. aureus* were, respectively, rifampin=40 minutes and rifamycin S=40 minutes; rifampin=40 minutes and rifamycin SV=20 minutes; rifampin=35 minutes and rifamycin B=20 minutes. In preliminary experiments, tolypomycin Y was found to kill phagocytosed *S. aureus* at initial rates too fast to measure at 10 μg/ml. Thus, rifampin and tolypomycin Y were compared at 10 and 1 μg/ml, respectively. In this experiment 50% destruction of phagocytosed *S. aureus* required approximately 60 minutes for rifampin and 50 minutes for tolypomycin Y. The results of comparing rifampin with rifamycin B and tolypomycin Y were selected for presentation (Fig. 3). To summarize these results, the rate of killing by rifampin was equaled by rifamycin S, and exceeded by rifamycin B, rifamycin SV, and especially by tolypomycin Y.

Comparisons of the Concentrations of Rifampin and Streptovaricins Required to Kill Phagocytosed *S. aureus*

In these experiments, conducted individually, the concentration of rifampin and the streptovaricins were varied while the incubation time was held constant at 90 minutes. The shape of the dose-response curves was generally the same for all compounds. Rifampin appeared to be more potent when compared to all streptovaricins. The results with streptovaricins A, B, C and D were selected for presentation (Fig. 4). The concentrations at which *ca.* 50% of the maximum killing occurred was for rifampin, 0.1 μg/ml; streptovaricin A, 0.2 μg/ml; streptovaricin B, 0.3 μg/ml; streptovaricin C, 0.3 μg/ml; streptovaricin D, 0.2 μg/ml; and streptovaricin G, 0.2 μg/ml. Analyzed in this fashion, rifampin was only 2 to 3 times more potent than the streptovaricins.

When the data analysis was made in terms of the total killing differential, the killing was, respectively for rifampin, 5-fold (average of 11 experiments); streptovaricin A, 5-fold; streptovaricin B, 3-fold; streptovaricin C, 3-fold; streptovaricin D, 3-fold; and streptovaricin G, 5-fold. Although the criterion

Fig. 4. The killing of phagocytosed *S. aureus* as a function of the concentration of streptovaricins A, B, C and D.

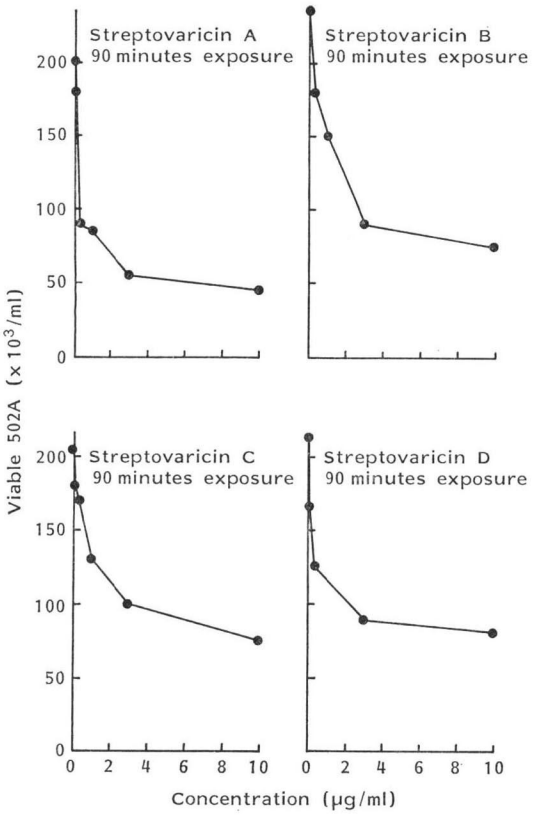
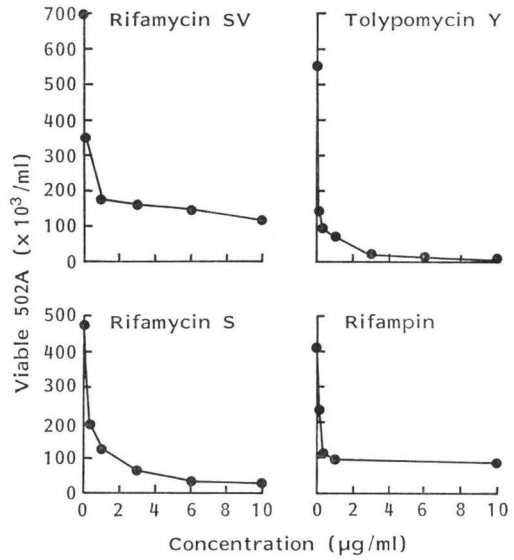


Fig. 5. The killing of phagocytosed *S. aureus* as a function of the concentration of rifamycin SV, tolypomycin Y, rifamycin S and rifampin.



for comparison is different, the results of these dose response experiments are in accord with the results of the rate of killing experiments described earlier. Thus, there was little difference between rifampin and the streptovaricins when the compounds were tested at 10 μg/ml utilizing an incubation period of 90 minutes. It should be noted that these results would not be anticipated on the basis of the MIC values. The latter indicated that rifampin was at least 30 times more potent than streptovaricin G and 60 to 120 times more potent than the rest of the streptovaricins.

Comparisons of the Concentrations of Rifampin, Rifamycins, and Tolypomycin Y Required to Kill Phagocytosed *S. aureus*

In these experiments, conducted individually, the concentrations of the compounds were varied while the incubation time was held constant at 90 minutes. As was the case with the streptovaricins, killing was more efficient at lower concentrations. In contrast to the streptovaricins the rifamycins SV, S, B and tolypomycin Y were more potent than rifampin. Rifampin killed about 40% of the phagocytosed *S. aureus* at 0.1 μg/ml whereas greater than 50% destruction was observed at less than 0.1 μg/ml with rifamycin SV, rifamycin S and tolypomycin Y (Fig. 5). In addition, essentially all bacteria were killed by tolypomycin Y.

When the data analysis was conducted in terms of total killing differential, the killing at 10 μg/ml was, respectively, for rifampin, 5-fold; rifamycin SV, 6-fold; rifamycin S, 19-fold; rifamycin B, 12-fold; and tolypomycin Y, >100-fold. These data, like those obtained with the streptovaricins, would not be anticipated on the basis of MIC values. In the latter case, rifampin was 8 times more potent than rifamycin SV and rifamycin S, and 60 times more potent than rifamycin B and tolypomycin Y. Thus, the

ability of these compounds to kill phagocytosed *S. aureus* was unrelated to their ability to kill extracellular *S. aureus*.

Microscopic Examination of Naphthalenic Ansamycin-treated Leukocytes Containing Phagocytosed *S. aureus*

When it became apparent that there was little correlation between the MIC data and the ability of a given naphthalenic ansamycin to kill phagocytosed *S. aureus*, the question of naphathalenic ansamycin toxicity to the leukocyte was considered. The specific question considered was the possibility that the naphthalenic ansamycins lysed the leukocytes resulting in exposure of the phagocytosed *S. aureus* to extracellular antibiotic. This artifact would cause serious misinterpretation of the experimental findings, since it would appear that the bacterial population had been reduced through antibiotic penetration of the phagocytes. However, microscopic examination of the leukocytes containing phagocytosed *S. aureus* after 2 hours of incubation with 10 μg/ml of the naphthalenic ansamycins tested here yielded the following results: There was no change in the number of leukocytes over the 2-hour incubation period and the leukocytes appeared normal under phase contrast microscopy.

The Effect of Warfarin and Other Coumarins on Rifampin's Killing of Phagocytosed *S. aureus*

The finding that many naphthalenic ansamycins could penetrate phagocytes and assist in killing intracellular staphylococci was unanticipated. The idea emerged that perhaps these compounds resembled a naturally occurring substance that was readily taken up by phagocytes. Of the few natural compounds containing the naphthalenic moiety, vitamin K (Fig. 1) seemed to be the most probable one having this property and was thus considered.

To explore the possibility that naphthalenic ansamycins may be entering phagocytes *via* a vitamin K transport system, the effect of a number of naphthaquinones on rifampin killing of phagocytosed *S. aureus* was studied. These included water soluble 2-hydroxy-1,4-naphthoquinone and menadione bisulfite, as well as a vitamin K colloidal suspension. However, none affected rifampin killing.

Nevertheless warfarin (Fig. 1), a well-known antagonist of vitamin K, clearly inhibited the ability of rifampin to kill phagocytosed *S. aureus*. Using 10 μg rifampin per ml, the viable count of phagocytosed *S. aureus* was reduced from 400×10^3 to 85×10^3 during a 90-minute incubation (Fig. 6). In the presence of warfarin, however, the killing activity of rifampin was diminished proportionately with increasing concentrations of warfarin so that full protection against rifampin killing was obtained at a warfarin concentration of 1,000 μg/ml (warfarin alone, at 1,000 μg/ml,

Fig. 6. The effect of warfarin on the killing of phagocytosed *S. aureus* by rifampin 10 μg per ml.

In the absence of rifampin and warfarin, the control cfu count was 400×10^3, the same count as that obtained with 10 μg rifampin per ml and 1,000 μg warfarin per ml.

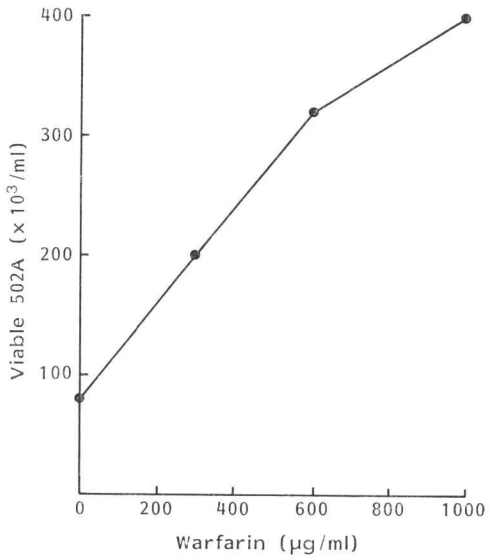


Fig. 7. The effect of novobiocin on the killing of phagocytosed *S. aureus* by rifampin at 10 μg per ml.

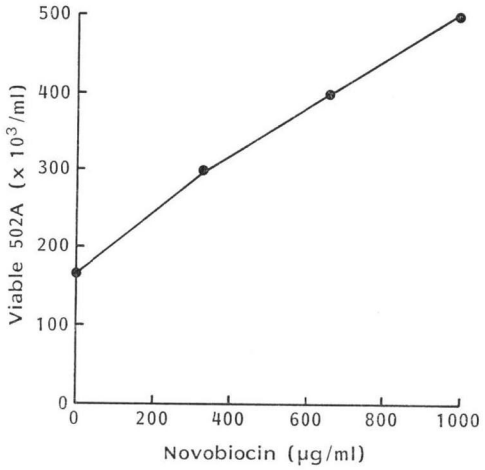
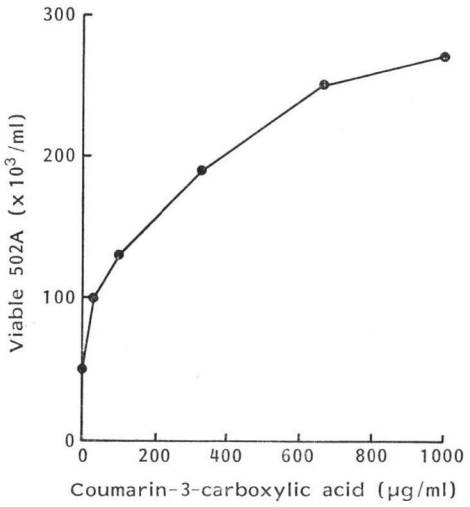


Fig. 8. The effect of coumarin-3-carboxylic acid on the killing of phagocytosed *S. aureus* by rifampin at 10 μg per ml.



did not affect leukocyte phagocytosis and killing of *S. aureus* 502A).

To rule out a direct competitive effect, rifampin and warfarin were mixed together and studied for extraphagocytic antimicrobial activity; warfarin had no effect on rifampin's anti-*S. aureus* activity at warfarin concentrations up to 800 μg per ml.

If warfarin had been toxic to leukocytes containing phagocytosed *S. aureus*, it would be reasonable to expect an enhancement of rifampin activity because of increased exposure to rifampin; instead, the opposite was observed. In addition, warfarin was not cytotoxic to mammaliam L1210 cells grown in culture at >200 μg warfarin per ml. Thus, the reversing effects of warfarin on rifampin's killing of phagocytosed *S. aureus* appear to be exerted directly on the leukocyte.

The studies with warfarin were extended to other coumarins including novobiocin and coumarin-3-carboxylic acid (Fig. 1). In these experiments rifampin was employed as before to kill up to 90% of the phagocytosed staphylococci. When these coumarins were added in the manner of warfarin at concentrations up to 1 mg per ml, *ca.* two-thirds of the phagocytosed bacteria were protected from the action of rifampin (Figs. 7 and 8).

This finding with novobiocin is also of interest from another perspective. Novobiocin is an antibiotic with roughly identical *in vitro* activity vs. extracellular *S. aureus* as rifampin. It is of extreme interest that novobiocin at concentrations *ca.* three orders of magnitude greater than its MIC vs. this organism has no destructive effect against phagocytosed staphylococci. In fact it protects them from rifampin. This finding distinctly illuminates differences between antibiotics concerning their abilities to kill intracellular pathogens.

Discussion

The most surprising finding in these studies was the lack of correlation between the ability of a given naphthalenic ansamycin to kill extracellular *S. aureus* compared with its ability to kill phagocytosed *S. aureus*. Rifampin was by far the most potent extracellular anti-staphylococcal agent of the compounds

tested, as much as 60 to 120 times more potent than some of the streptovaricins and tolypomycin Y. Yet, there were only minimal differences between the streptovaricins and rifampin in the killing of phagocytosed *S. aureus*. In addition, several rifamycins and especially tolypomycin Y were even more effective than rifampin in killing phagocytosed *S. aureus*. These unanticipated results were true using several criteria of comparison: The rate of bacterial killing, the total number of bacteria killed, or the concentration at which 50% of the bacteria were killed. The studies of GEMMELL[17] may be instructive concerning this lack of correlation. Bacteria when treated with certain antibiotics at concentrations well below their MIC's are often altered making them more susceptible to phagocytic destruction. In the experiments reported here, it is possible that only sub-MIC levels of the naphthalenic ansamycins are required to prime the intracellular staphylococci for phagocytic destruction. Thus the differences in MIC between these antibiotics may not be of prime importance.

There are no simple explanations as to why certain antibiotics may penetrate phagocytes. As BROWN and PERCIVAL[15] noted, these factors can include diffusion rates, molecular size, protein binding, dissociation constants, or lipid solubility. Indeed, lipid solubility was the reason originally suggested[2,3,4] for the ability of rifampin to penetrate phagocytes. Similar arguments have been presented for chloramphenicol activity against phagocytosed *Haemophilus influenzae* type b[19]. However, this explanation obviously does not apply to water-soluble antibiotics that penetrate phagocytes, *e.g.*, the destructive effects of penicillin against phagocytosed gonococci[20,21], streptomycin against phagocytosed *S. aureus*[6], and clindamycin penetration into phagocytes[22,23,24].

Perhaps the most forceful argument against leukocyte penetration of rifampin *via* simple lipid solubility comes from the work of PESANTI[25]. When mouse macrophages were employed as the phagocyte, rifampin was unable to penetrate and kill phagocytosed *S. aureus*. This implies a level of specificity greater than just solubility in a phagocyte plasma membrane. Also consistent with this argument are the current data with sodium warfarin which is water-soluble yet readily inhibited rifampin's effects on phagocytosed *S. aureus*.

When it became apparent that many naphthalenic ansamycins were part of the select group of antibiotics that can rapidly kill phagocytosed bacteria, several hypotheses were made to explain their unique properties. Because of structural similarities, it was first considered that the naphthalenic ansamycins may be entering phagocytes by mechanisms involved in vitamin K transport. In addition, not only are there structural similarities, but the biosyntheses of vitamin K and the rifamycins partially involve common enzymes[26]. Attempts were made to reverse naphthalenic ansamycin effects with vitamin K preparations, but no reversing effects were observed. However, a well-known vitamin K antagonist, warfarin[27], was able to totally reverse the intraphagocytic killing by rifampin. It may also be relevant that warfarin and rifampin appear to be antagonistic in humans; the dosage of warfarin had to be tripled in the presence of rifampin[28]. However, the evidence for naphthalenic ansamycins entering *via* a vitamin K transport is, for the moment, indirect evidence.

The possibility that naphthalenic ansamycins may be entering *via* a specific phagocyte receptor mechanism also should be considered. One candidate is the receptor for synthetic chemotactic peptides[29~34]. Rifampin blocks the chemotactic response to these peptides but not to serum-derived chemoattractants[35,36,37]. Furthermore, rifampin blocks the binding of radiolabelled *N*-formylmethionylleucylphenylalanine (R. D. NELSON, personal communication) but not that of radiolabelled C5a (D. CHENOWETH, personal communication). This implies that rifampin is indeed binding to certain specific chemotactic receptors. However, there is no obvious structural similarity between the naphthalenic ansamycins and the synthetic chemotactic peptides; therefore, the rather specific effects of rifampin may be on a site adjacent to the synthetic chemotactic peptide receptor.

In considering the core portion of the naphthalenic ansamycins that may be necessary for phagocyte penetration, the studies of STYRT *et al.*[38] are instructive. They showed that radiolabelled daunomycin was rapidly concentrated inside human leukocytes. Although daunomycin contains a 4 ring structure of the anthracycline class, the middle two rings closely resemble the fused phenolic, bicyclic system of the streptovaricins, rifamycins, and rifampin. Thus, the fused phenolic bicyclic system common to all the naphthalenic ansamycins is tentatively considered to be the portion necessary for phagocyte penetration.

The bicyclic system may also explain the blocking effects of novobiocin on rifampin's intracellular

killing. Structural models of rifampin, streptovaricin A and novobiocin showed many similarities concerning their bicyclic rings. As novobiocin was shown in other experiments to readily bind to polymorphonuclear neutrophils, the oxygen in its coumarin ring may account for its failure to enter leukocytes and to block such entry by rifampin.

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