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THE EFFECT OF DIHYDROFOLATE REDUCTASE INHIBITORS ON *Mycobacterium Leprae* IN THE MOUSE FOOT PAD

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McCullough and Maren(1) have demonstrated in *Escherichia coli* that, like sulfonamides, dapsone acts by inhibiting the incorporation of para-aminobenzoic acid into dihydrofolate. This essential step in folate biosynthesis is required by bacterial cells, while higher organisms rely on food sources for preformed folates. The next step in the folic acid pathway, the enzymatic reduction of dihydrofolic acid to the active form tetrahydrofolic acid, is shared by bacterial and mammalian cells. Tetrahydrofolic acid acts as a carrier of one-carbon fragments crucial to a battery of essential biosynthetic reactions, including the synthesis of purines, serine, methionine and thymine. The synthesis of thymine is exceptional in that, in the process of performing one carbon transfer, tetrahydrofolate is simultaneously oxidized to the dihydro derivative which again requires enzymatic reduction to the active moiety, tetrahydrofolate(2).

Dihydrofolate reductase inhibitors have been synthesized that bind differentially to bacterial and not mammalian enzymes. One of these, trimethoprim, has proved to be a potent antimicrobial. By virtue of sequential blockade of folic acid synthesis, trimethoprim and sulfamethoxazole together act synergistically against a wide variety of aerobic gram positive and gram negative bacteria. This synergism can be demonstrated *in vitro* by a reduction in the minimal inhibitory concentration of each drug and by an increase in bactericidal activity(2). Combination therapy with trimethoprim and sulfamethoxazole has proved useful clinically, particularly against urinary pathogens.

Dihydrofolate reductase inhibitors in general have not been effective against *M. tuberculosis*. Shepard(3) found trimethoprim ineffective against *M. leprae* in the mouse foot pad and incapable of potentiating the effect of dapsone. Morrison(4) found that trimethoprim was a poor inhibitor of dihydrofolate reductase from *M.* sp 607 and only a moderate growth inhibitor. Morrison screened a group of dihydrofolate reductase inhibitors that were 2,4-diamino-6-substituted pyridines against *M.* sp 607. This group of compounds proved excellent as enzyme inhibitors but had large minimal inhibitory concentrations, suggesting poor penetration of the mycobacterial cell wall. Morrison(5) also tested another series of dihydrofolate reductase inhibitors, 2,4-diaminoquinazolines. The activity of this series

of compounds against nonmycobacterial organisms was not impressive. However, they were ten-fold more tightly bound to the dihydrofolate reductase from M. sp 607 than were the previously tested pyridines. Most importantly, they proved moderate to strongly effective as growth inhibitors of M. sp 607 and in combination with dapsone pronounced synergism could be demonstrated. In combination, the M.I.C. of the quinazoline was reduced by eight-fold and of dapsone by 20-fold.

Because of these encouraging data obtained from M. sp 607, we designed studies to test the efficacy against M. leprae in the mouse foot pad of certain of these quinazolines alone and in combination with DDS. Certain other dihydrofolate reductase inhibitors were also studied.

In the first set of studies, BALB/c male mice were fed diets containing 0.01 g % of various dihydrofolate reductase inhibitors from day 60 to day 150 after foot pad infection with 5×10^3 M. leprae. In the first table are depicted the basic quinazoline structure and the compounds tested. The first nine compounds were synthesized by Dr. Joseph DeGraw of the Stanford Research Institute and include seven 2,4-diaminoquinazolines. The last three compounds, including one quinazoline, were supplied by Dr. Robert S. Rozman of the Walter Reed Army Institute of Research.

The results of this screening can be found in the first figure. Ten of the twelve drugs were found to exert a bacteriostatic effect -- that is, multiplication of M. leprae was inhibited during the period of drug administration but resumed promptly upon withdrawal of drug. SRI 93, 2,4-diaminoquinazoline itself, and SRI 91, 5-methyl-6-n-hexyl-2,4-diaminoquinazolines, were inactive. The other six quinazolines were all active, but not as active as was 0.0001% dapsone. From these initial studies, a structure-activity relationship has emerged for the 2,4-diaminoquinazoline derivatives. Methyl-substitution at position #5 is essential for activity; substitution also at position #6 with a short aliphatic chain, c_1 to c_4, increased activity, whereas substitution in this position with an n-hexyl chain appears to have abolished activity.

Next we studied more extensively in the mouse foot pad system two of the active quinazolines, the 5-methyl-6-n-propyl-2,4-diaminoquinazoline and the 5,6-dimethyl-2,4-diaminoquinazoline. These compounds were studied at varying dose levels in an attempt to define their minimal inhibitory concentrations and in combination with dapsone to ascertain if synergism could be established. In the second table are presented the drug concentrations that were fed mice between day 60 and 150 after foot pad infection with 5×10^3 M. leprae.

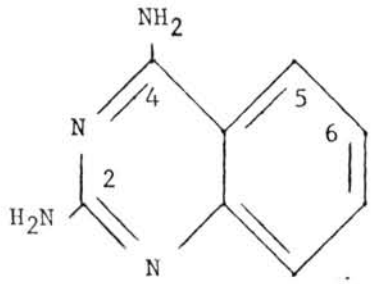
In Figures 2 and 3 are presented the resultant growth curves. Certain exciting and perplexing features are evident. Once again these quinazolines alone appear active against M. leprae in the mouse foot pad,

although less so than in the original screen. As in the original screen, they permit multiplication but rather uniformly to lower levels than untreated controls. Lower concentrations of these quinazolines appear generally more active than higher concentrations. Because growth curves of M. leprae from quinazoline treated and untreated mice are parallel from the time of the first harvests, those from the treated animals merely beginning from lower concentrations of M. leprae, it appears that the quinazolines exert an early antibacterial effect that has waned by the time of the first harvest. These studies do not suggest whether this early effect is bacteriostatic or bactericidal. If indeed an early bactericidal effect had occurred, the similarity of the growth curves in untreated animals inoculated with 500 M. leprae to those in mice inoculated with 5000 organisms and treated with 0.001% of the 5-methyl-6-n-propyl derivative implies 90% bacteriocide.

Both quinazolines in combination with dapsone permitted some multiplication prior to the first harvest, although to generally lower levels than dapsone alone. From the time of the first harvest during continued drug administration, it appears that both quinazolines combined with dapsone resulted in increased antibacterial activity as compared to dapsone alone. Furthermore, during this later period of drug administration, this combination therapy appears to have produced killing of M. leprae rather than the apparent bacteriostasis produced by dapsone alone. Curiously, this synergism occurs late in the course of drug administration when the activity of the quinazoline alone is no longer apparent. Also, as was the case during quinazoline monotherapy, lower concentrations of both quinazolines were more active in combination with dapsone than were higher concentrations. When combined therapy is discontinued, growth curves appear to approach those of dapsone alone. Clearly many questions remain to be answered about these most interesting and promising compounds.

Although dapsone has proved efficacious in the treatment of multibacillary leprosy, the twin problems of drug resistance and bacterial persistence demand a continued search for new chemotherapeutic agents. Despite the clearly much greater bactericidal activity of rifampicin, its expense and seeming inability to prevent bacterial persistence(6) do not diminish the need for other antimicrobials active against M. leprae. As a class, the dihydrofolate reductase inhibitors offer much promise: they may be used in combination with sulfones to prevent the emergence of drug resistance; as trimethoprim is active and even synergistic with sulfonamides against sulfonamide resistant bacteria(7), combinations of a sulphone plus a dihydrofolate reductase inhibitor may prove useful in treating sulphone resistance in leprosy. In these respects, the dihydrofolate reductase inhibitors may have an additional advantage, namely low cost, which has been the major drawback to the widespread use of rifampicin for such purposes. Recently, short course therapeutic regimens for pulmonary tuberculosis have dealt effectively with bacterial persistence simply by the inclusion of two bactericidal agents acting through different

TABLE 1: DIHYDROFOLATE REDUCTASE INHIBITORS SCREENED



Basic 2,4-diaminoquinazoline structure

SRI 19	--	6-isobutyl-2,4-diaminopteridine
SRI 104.6	--	8-deazapteroic acid ethyl ester
SRI 93	--	2,4-diaminoquinazoline
SRI 90	--	5-methyl-2,4-diaminoquinazoline
SRI 68	--	5,6-dimethyl-2,4-diaminoquinazoline
SRI 105	--	5-methyl-6-n propyl-2,4-diaminoquinazoline
SRI 126	--	5-methyl-6-n butyl-2,4-diaminoquinazoline
SRI 91	--	5-methyl-6-n hexyl-2,4-diaminoquinazoline
SRI 151.4	--	5-methyl-6-nitro-2,4-diaminoquinazoline
WRAIR-BC	--	6-(3-trifluoromethylphenylthio)-2,4-diamino-quinazoline
WRAIR-BD	--	a 5-substituted 2,4-diaminopyrimidine
WRAIR-ZB	--	6-(2-methylphenyl)-2,4,7-triaminopteridine

TABLE 2: MOUSE DIETS

2,4-diaminoquinazoline	0.01 %
2,4-diaminoquinazoline	0.003 %
2,4-diaminoquinazoline	0.001 %
2,4-diaminoquinazoline	0.0003%
DDS	0.0001%
2,4-diaminoquinazoline + DDS	0.01 % 0.0001%
2,4-diaminoquinazoline + DDS	0.03 % 0.0001%
2,4-diaminoquinazoline + DDS	0.001 % 0.0001%

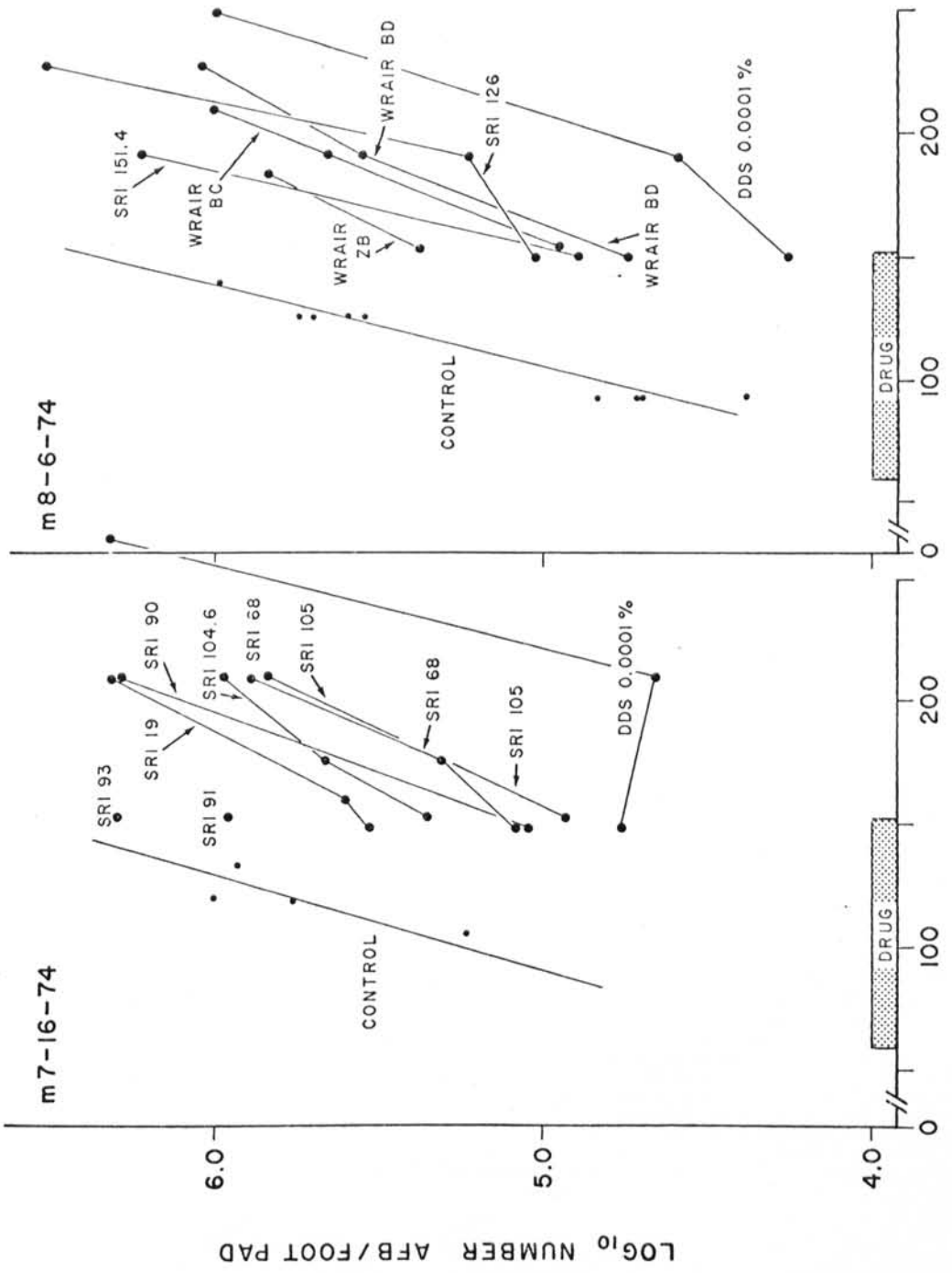

m8-6-74
m7-16-74
SRI 151.4
WRAIR BC
WRAIR ZB
WRAIR BD
SRI 126
DDS 0.0001 %
CONTROL
SRI 93
SRI 19
SRI 90
SRI 91
SRI 104.6
SRI 68
SRI 105
DRUG
0
100
200
6.0
5.0
4.0
LOG10 NUMBER AFB/FOOT PAD
TIME AFTER INOCULATION (Days)

FIG. 1.

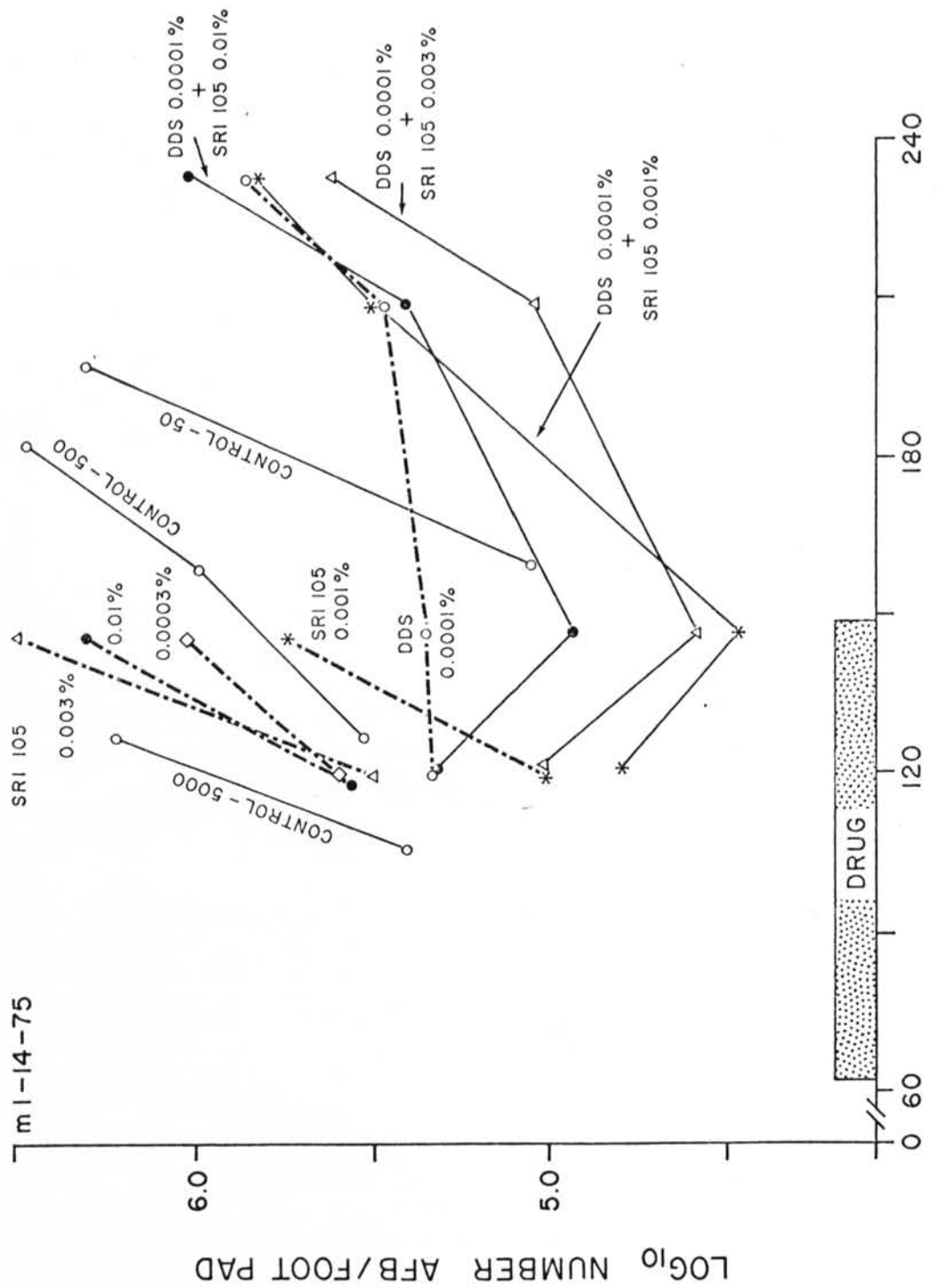

DDS 0.0001% + SRI 105 0.01%
DDS 0.0001% + SRI 105 0.003%
DDS 0.0001% + SRI 105 0.001%
CONTROL - 50
CONTROL - 500
CONTROL - 5000
SRI 105 0.01%
SRI 105 0.003%
SRI 105 0.001%
DDS 0.0001%
DRUG
m1-14-75
6.0
5.0
LOG10 NUMBER AFB/FOOT PAD
0 60 120 180 240
TIME AFTER INOCULATION (days)

FIG. 2.

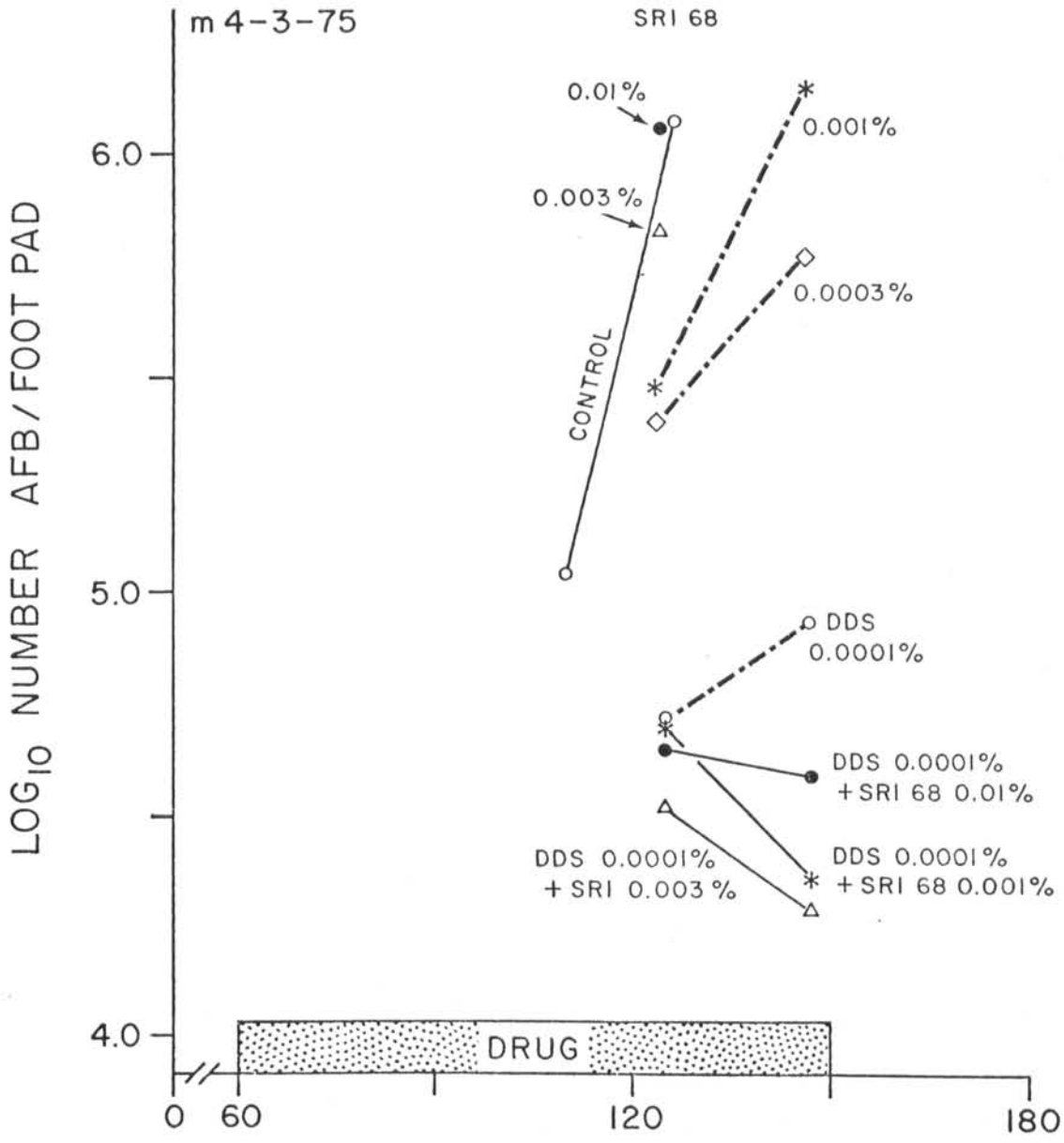

m 4-3-75
SRI 68
0.01%
0.001%
0.003%
0.0003%
CONTROL
DDS
0.0001%
DDS 0.0001%
+SRI 68 0.01%
DDS 0.0001%
+ SRI 0.003%
DDS 0.0001%
+ SRI 68 0.001%
DRUG
6.0
5.0
4.0
0
60
120
180
LOG10 NUMBER AFB/FOOT PAD
TIME AFTER INOCULATION (days)

FIG. 3.

mechanisms. This has reduced the duration necessary for effective therapy from 2 years to 6 months(8). In the treatment of lepromatous leprosy, bacterial persistence, which results in the requirement for lifelong therapy, might similarly be minimized if dapsone and a dihydrofolate reductase inhibitor were found sufficiently bactericidal and these were combined with rifampicin, providing in leprosy the necessary two bactericidal agents.

In summary, we have screened a number of dihydrofolate reductase inhibitors against M. leprae in the mouse and found them to be active. From the screening of these compounds, a structure activity relationship for the 2,4-diaminoquinazolines has emerged. Initial studies with two 2,4-diaminoquinazolines in combination with dapsone have shown an increased antibacterial effect as compared to DDS alone. Certainly these compounds require continued research and development.

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