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New *Mycobacterium avium* Antifolate Shows Synergistic Effect when Used in Combination with Dihydropteroate Synthase Inhibitors

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Mycobacterium avium **complex (MAC) is resistant to trimethoprim, an inhibitor of bacterial dihydrofolate reductase (DHFR). A previously identified selective inhibitor of MAC DHFR, SRI-8858, was shown to have synergistic activity in combination with dapsone and sulfamethoxazole, two drugs that inhibit bacterial dihydropteroate synthase.**

Effective chemotherapy of patients coinfected with *Mycobacterium avium* complex (MAC) and human immunodeficiency virus is difficult, primarily because MAC is resistant to a variety of antimycobacterial agents. Although some success has been achieved through the rational use of multiple-drug combination therapy, the resistant nature of MAC emphasizes the need for new drugs.

Previously, we identified a specific group of 2,4-diamino-5-methyl-5-deazapteridines (DMDPs) that are active against MAC. Demonstration of the antimycobacterial activity of these new antifolates was initially aided by efforts of the National Institutes of Health-sponsored Tuberculosis Antimicrobial Acquisition and Coordinating Facility. Continued efforts have resulted in a specific group of DMDPs that have selective activity for MAC dihydrofolate reductase (DHFR) but not human DHFR, which makes them good candidates for further development (7). This is significant because MAC is intrinsically resistant to trimethoprim, a commonly used drug that targets prokaryotic DHFRs but not human DHFR. We have shown that the 50% inhibitory concentration of trimethoprim for the MAC DHFR is 4,100 nM, in comparison to the new DMDPs, which have 50% inhibitory concentrations around 1.0 nM (7). DHFR is a key enzyme in the folate biosynthetic pathway that catalyzes the reduction of dihydrofolate to tetrahydrofolate, derivatives of which function in single carbon transfers at various oxidation states for the synthesis of purines, methionine, glycine, pantothenate, thymidylate, and *N*-formylmethionyl-tRNA (3, 5). Inhibition of DHFR leads to a depletion of tetrahydrofolate derivatives and results ultimately in inhibition of DNA, RNA, and protein synthesis (3, 5).

Trimethoprim is generally used in combination with sulfamethoxazole (SMX) to treat infections caused by susceptible organisms. Sulfa drugs such as SMX inhibit another enzyme in the folate pathway, dihydropteroate synthase (DHPS). A dual blockage in the pathway is believed to be responsible for a synergistic increase in activity seen with the drug combination (4).

The objective of the present study was to evaluate the activity of a new DMDP, SRI-8858 (Fig. 1), in combination with SMX and dapsone, two known inhibitors of bacterial DHPS. This will hopefully shed some light on the ability of these new DMDP derivatives to act in combination with clinically acceptable DHPS inhibitors and aid in the development of dual drug therapy for MAC infections.

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SRI-8858 {6-[2,5-diethoxyphenylamino)methyl]-2,4-diamino-5-methylpyrido[2,3-*d*]pyrimidine} is the hydrochloride salt form of SRI-8686, a DMDP derivative whose synthesis has been described previously (7). Sulfamethoxazole and dapsone were purchased from Sigma. All drugs were dissolved in dimethyl sulfoxide at 10.24 mg/ml and stored frozen at −80°C. For assay, serial twofold dilutions were made in assay medium (see below), with the final dimethyl sulfoxide concentration being 1.3%.

The MAC strains used for these studies were NJ168 (serovar 1), NJ211 (serovar 4/6), and NJ3404 (serovar 4), kindly supplied by L. Heifets, National Jewish Center for Immunology and Respiratory Diseases, Denver, CO. These strains were used previously by us to show selective activity against MAC of the DMDP derivatives (7).

Antimicrobial activity was evaluated with a colorimetric broth microdilution assay as reported previously (1, 6, 7) except for the medium composition. Frozen broth cultures were thawed, diluted in assay medium (Middlebrook 7H9 broth supplemented with 0.2% glycerol, albumin-dextrose-catalase enrichment, 0.1% Casamino Acids, 0.001 mg/ml pantothenate, and 0.02 mg/ml adenine) to about 2×10^5 CFU/ml, and used as the inoculum. Drug dilutions (0.05 ml/well) at twice the desired concentration were added to appropriate wells followed by 0.05 ml of inoculum. Viability controls and uninoculated drug and medium controls were included with each assay. The plates were incubated at 37°C for 6 days, at which time the redox indicator alamarBlue (Trek Diagnostic Systems) was added to each well as a mixture with Tween 80. Incubation was continued for 18 to 22 h. The plates were read in an optical microtiter plate reader programmed to subtract the absorbance at 600 nm from that at 570 nm to blank out turbidity and absorbance due to oxidized dye. The MIC was reported as the

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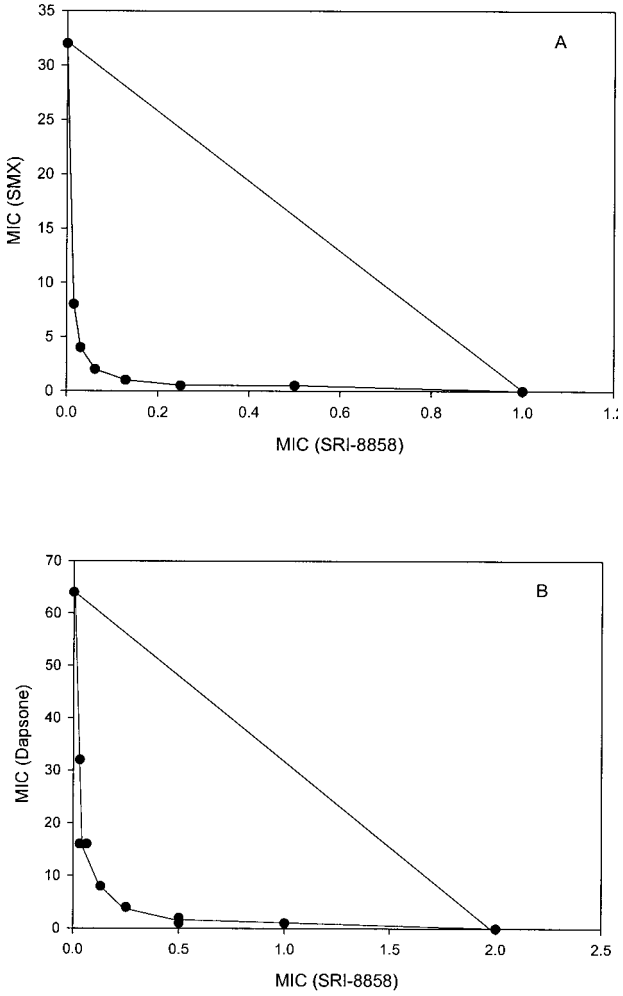

FIG. 1. Isobolograms of SRI-8858–SMX (A) and SRI-8858–dapsone (B) combinations against MAC NJ168. The MICs of SRI-8858 are 1.0 μg/ml in panel A and 2.0 μg/ml in panel B. The MIC of SMX is 32 μg/ml (A), and the MIC of dapsone is 64 μg/ml (B).

lowest concentration of drug yielding a differential absorbance of zero or less. For MAC NJ168, MAC NJ211, and MAC NJ3404, the MICs of SRI-8858 were 1.0 to 2.0, 0.25, and 0.13 to 0.5 μg/ml, respectively. For the same three MAC strains, the MICs of SMX were 32, 32, and >64 μg/ml, respectively, and the MICs of dapsone were 64, 64, and 32 μg/ml, respectively.

For combination studies, SRI-8858 was tested with either SMX or dapsone using a checkerboard format and the broth microdilution assay described above. Assays with MAC NJ168 were performed in duplicate. Results from each drug combination were interpreted according to an interaction index (2). This was calculated as the fractional inhibitory concentration (FIC), which is the ratio of the MIC of each drug in combination to the MIC of that drug when used alone. The activity of the combination was considered synergistic if the sum of the FICs for a combination was less than 0.5. A sum of about 1 is additive, and a sum of >1 is indicative of antagonism. The diagonal line on the isobologram represents an additive effect; a point below this line represents synergism and a point above this line represents antagonism. The results are presented in Tables 1 to 4 and the accompanying isobolograms (Fig. 1).

As shown in Fig. 1, the combination of SRI-8868 with SMZ or dapsone is synergistic for MAC NJ168. This synergism was also observed with MAC NJ211 and NJ3404 (Tables 1 through 4). The FICs for the MAC strains, at all concentrations of drugs, were 0.5 or below. Maximum synergism for NJ168 was observed when SRI-8858 was at 0.063 μg/ml and SMX was at 2.0 μg/ml (Fig. 1). Maximum synergism for NJ211 was observed when SRI-8858 and SMX were at 0.016 and ≤0.25 μg/ml, respectively (Table 1). Maximum synergism for NJ3404 was observed when SRI-8858 was at 0.016 μg/ml and SMX was at ≤0.25 μg/ml, respectively (Table 2).

With regard to SRI-8858 and dapsone, synergism was also noted with this combination of drugs (Fig. 1B and Tables 3 and

TABLE 1. SRI-8858–SMX combination against MAC NJ211

MIC (μg/ml) of SRI-8858[a]	FIC of SRI-8858	MIC (μg/ml) of SMX	FIC of SMX	FIC sum
0.25	1	0	0	1
0.13	0.5	≤0.25	0.008	0.51
0.063	0.25	≤0.25	0.008	0.26
0.031	0.13	≤0.25	0.008	0.14
0.016	0.063	≤0.25	0.008	0.071
≤0.016	0.063	1.0	0.031	0.094
≤0.016	0.063	4	0.13	0.19
≤0.016	0.063	8	0.25	0.31
0	0	**32**	1	1

[a] Bold numbers are the MICs of the given drugs.

TABLE 2. SRI-8858–SMX combination against MAC NJ3404

MIC (μg/ml) of SRI-8858[a]	FIC of SRI-8858	MIC (μg/mL) of SMX	FIC of SMX	FIC sum
0.5	1	0	0	1
0.25	0.5	≤0.25	0.004	0.5
0.13	0.25	≤0.25	0.004	0.25
0.063	0.13	≤0.25	0.004	0.13
0.031	0.063	≤0.25	0.004	0.067
0.016	0.031	≤0.25	0.004	0.035
≤0.016	0.031	1	0.016	0.047
≤0.016	0.031	4	0.063	0.094
≤0.016	0.031	8	0.13	0.16
≤0.016	0.031	16	0.25	0.28
0	0	**>64**	1	1

[a] Numbers in bold are the MICs of the given drugs.

TABLE 3. SRI-8858–dapsone combination against MAC NJ211

MIC (μg/ml) of SRI-8858[a]	FIC of SRI-8858	MIC (μg/ml) of dapsone	FIC of dapsone	FIC sum
0.25	1	0	0	1
0.13	0.5	≤0.25	0.004	0.5
0.063	0.25	≤0.25	0.004	0.25
0.031	0.13	0.5	0.008	0.14
0.016	0.063	1	0.016	0.079
≤0.016	0.063	4	0.063	0.13
≤0.016	0.063	16	0.25	0.31
0	0	**64**	1	1

[a] Bold numbers are the MICs of the given drugs.

TABLE 4. SRI-8858–dapsone combination against MAC NJ3404

MIC (μg/ml) of SRI-8858[a]	FIC of SRI-8858	MIC (μg/ml) of dapsone	FIC of dapsone	FIC sum
0.13	1	0	0	1
0.063	0.5	≤0.25	0.008	0.5
0.031	0.25	≤0.25	0.008	0.26
0.016	0.13	0.5	0.016	0.15
≤0.016	0.13	2	0.063	0.19
≤0.016	0.13	8	0.25	0.38
0	0	**32**	1	1

[a] Bold numbers are MICs of the given drugs.

4). The maximum synergism for NJ168 was obtained when SRI-8858 was within the range of 0.25 and 0.13 μg/ml and dapsone was within the range of 4 and 8 μg/ml (Fig. 1B). Maximum synergism for NJ211 was obtained with SRI-8858 at 0.016 and dapsone at 1.0 μg/ml (Table 3). For NJ3404, the maximum synergism was noted when SRI-8858 was 0.016 and dapsone was 0.5 μg/ml (Table 4).

These results demonstrate the utility of using a sulfonamide in combination with the new antifolate SRI-8858. In all three MAC strains, a synergistic effect was observed with both SMX and dapsone. For SRI-8858–SMX combinations, synergism was observed in all of the 22 combinations tested. For SRI-8858–dapsone combinations, synergism was observed with all of the 17 combinations tested. We understand that in vitro methods used to predict in vivo activity (e.g., the FIC method) are not highly reliable without some knowledge of the pharmacologic parameters of the drug. However, our results are preliminary and of sufficient interest to warrant further study.

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