Synergistic Combinations of Ro 11-8958 and Other Dihydrofolate Reductase Inhibitors with Sulfamethoxazole and Dapsone for Therapy of Experimental Pneumocystosis

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We compared Ro 11-8958, an analog of trimethoprim (TMP) with improved antimicrobial and pharmacokinetic properties, other dihydrofolate reductase (DHFR) inhibitors, sulfamethoxazole (SMX), and dapsone (DAP) in the treatment of Pneumocystis carinii pneumonia in an immunosuppressed rat model. In contrast to previous reports, high dosages of the DHFR inhibitors were used in combination with fixed, low dosages of SMX (3 mg/kg of body weight per day) or DAP (25 mg/kg/day). When administered alone at these dosages, SMX and DAP reduced the median P. carinii cyst count about 5- to 15-fold. Ro 11-8958, TMP, and diaveridine used at a dosage of 20 mg/kg/day with SMX were only slightly more effective than SMX used alone. However, administration of these DHFR inhibitors at a dosage of 100 mg/kg/day with SMX lowered the cyst count about 500- to 1,000-fold, indicating a synergistic effect. Little or no synergism was found when other DHFR inhibitors (pyrimethamine, cycloguanil, and tetroxoprim) were combined with SMX. Regimens of Ro 11-8958 at a dosage of 20 mg/kg/day with DAP and of TMP or diaveridine used at a dosage of 100 mg/kg/day with DAP showed comparable anti-P. carinii activity, lowering the cyst count 100- to 200-fold. By contrast, Ro 11-8958 administered at a dosage of 100 mg/kg/day with DAP reduced the cyst count >1,000-fold. Thus, the experimental approach used here enables the rat model of pneumocystosis to be used to compare synergistic combinations of antifolate drugs. The favorable results achieved with Ro 11-8958 indicate that it should be considered for clinical trials.

Drugs which inhibit folic acid synthesis have long been a mainstay in the treatment of Pneumocystis carinii pneumonia (12). The fact that these agents have only a static effect on P. carinii (6) and frequently cause adverse effects in human immunodeficiency virus patients (5, 15, 23) has emphasized the need for new compounds. It has been assumed that the antifoate combination of dihydrofolate reductase (DHFR) inhibitor and sulfonamide or sulfone acts synergistically against P. carinii in a manner similar to that against bacteria or other microbes; however, definitive information is lacking. Limited studies have shown that the use of these agents alone in the treatment of pneumocystosis in humans is associated with a suboptimal response or high rate of relapse (1, 14). The most widely used drug combination has been the DHFR inhibitor trimethoprim (TMP), used at a fixed dose with sulfamethoxazole (SMX). Other combinations have included TMP and dapsone (DAP) (13), pyrimethamine (PYR) and sulfadiazine (10), and trimetrexate and sulfadiazine (1). Controlled clinical trials directly comparing the efficacy and toxicity of different combinations or dosage regimens of these agents in the treatment of P. carinii pneumonia would be of interest.

Most studies of new anti-P. carinii drugs have been performed with animal models, particularly immunosuppressed rats, because of the lack of a reliable in vitro cultivation system (4, 7, 19). Compounds which show activity in these models are usually active against P. carinii in humans. In a previous report, we found that sulfonamides and sulfones used alone were highly effective over a wide range (60 to 500 mg/kg of body weight per day) of dosages in the treatment of pneumocystosis in the rat model (21). DHFR inhibitors used alone were ineffective and did not improve the activity of the sulfonamides or sulfones. Our more recent study has demonstrated that a dose-response curve with sulfonamides could be achieved when very low (0.3 to 15 mg/kg/day) doses were used (18). We hypothesized that the lack of synergism with DHFR inhibitors and sulfonamides or sulfones in the rat model might, in part, be due to selection of the incorrect dose.

We undertook the present study to determine whether high doses of DHFR inhibitors used in combination with a fixed, low dosage of a sulfonamide or sulfone might act synergistically in the treatment of pneumocystosis in immunosuppressed rats. The efficacy of Ro 11-8958, an analog of TMP with improved antimicrobial spectrum and pharmacokinetic properties (16), was also investigated in this animal model.

MATERIALS AND METHODS

Drugs. The structural formulas of the DHFR inhibitors used in this study are presented in Fig. 1. TMP, diaveridine (DIA), PYR, SMX, and DAP were purchased from Sigma Chemical Co. (St. Louis, Mo.). Ro 11-8958 was provided by Roy Cleeland (Hoffmann-La Roche, Inc., Nutley, N.J.) and R. L. Then and A. Polak (F. Hoffmann-La Roche, Ltd., Basel, Switzerland). Cycloguanil was provided by Dan Santi (University of California—San Francisco). Tetroxoprim was provided by Charles Litterst, Chris Lambros, and Barbara Laughon, Division of AIDS, National Institute of Allergy and Infectious Diseases. Folinic acid (FA) (calcium leucov-
orin) was purchased from Lederle Laboratories (Pearl River, N.Y.); FA was administered with PYR to prevent bone marrow toxicity while not interfering with its anti-\textit{P. carinii} activity (18, 21). Drugs which were to be administered orally were prepared as a 2% ethanol suspension. Drugs to be administered parenterally were dissolved in sterile water.

**Animal model.** The experimental protocol has been described in detail previously (18, 20–22). Adult male Sprague-Dawley rats, obtained from Sasco, Inc. (St. Louis, Mo.), and weighing about 150 g, with latent \textit{P. carinii} infection were used. The animals were housed in microisolator cages in a Biobubble (CSA Fluid Dynamics, Fort Collins, Colo.), which is a plastic unit with laminar air flow and HEPA filtration, and given autoclaved water, food, and bedding. After a period of acclimation, the rats were administered 4 mg of methylprednisolone acetate (Depo-Medrol; The Upjohn Co., Kalamazoo, Mich.) subcutaneously (s.c.) once weekly and 1 mg of cephradine (Velosef; E. R. Squibb, Princeton, N.J.) per ml in the drinking water to provoke the development of \textit{P. carinii} pneumonia while suppressing bacterial infection.

The rats were weighed and monitored, and sentinel animals were sacrificed periodically to check the progression of pneumocystosis. After about 6 weeks, when the infection reached moderate intensity, the rats were divided randomly into treatment and control groups of about 15 to 20 animals each. Drugs to be tested were given by oral gavage or parenterally in single or divided doses for 3 weeks, during which time the rats remained on the immunosuppressive regimen. Control animals receiving steroids (C/S group) were administered either no anti-\textit{P. carinii} drugs or a placebo. At the end of this time, the rats were sacrificed by an overdose of halothane anesthesia.

**Analysis of drug efficacy.** The procedures used to evaluate the anti-\textit{P. carinii} activity of the drugs have been reported in detail in our earlier studies (3, 9). Assessment of efficacy was
RAT GROUPS

FIG. 2. Treatment of pneumocystosis with TMP, Ro 11-8958, and SMX alone or in combination as assessed by the number of cysts per lung. The SMX dosage was 3 mg/kg/day per os (p.o.) unless designated otherwise. The p.o. dosage (indicated in parentheses in the figure) regimens used were TMP, 20 mg/kg/day with SMX; TMP, 100 mg/kg/day with SMX; TMP, 100 mg/kg/day; Ro 11-8958, 20 mg/kg/day with SMX; Ro 11-8958, 100 mg/kg/day with SMX; Ro 11-8958, 100 mg/kg/day; and SMX. TMP(50)-SMX(250), TMP at 50 mg/kg/day and SMX at 250 mg/kg/day p.o. (a control group receiving the standard treatment regimen); C/S, controls receiving steroids; C/N, control normal rats not receiving any medications. Horizontal bars indicate median values.

Based on the magnitude of pneumocystosis in the lungs rather than animal survival because the rats sometimes died of causes (e.g., other opportunistic infections or adverse drug reactions) unrelated to *P. carinii*. Rats had to receive at least 10 days of drug treatment to be included in the data analysis because it customarily takes this long to observe an anti-*P. carinii* effect.

The principal method of determining the magnitude of pneumocystosis and the effects of the drug treatment was the quantitation of the organism burden (18, 19). At death or sacrifice, the right lung was weighed, homogenized, and stained with cresyl echt violet for counting the number of *P. carinii* cysts; the lower limit of detection was $1.1 \times 10^5$ organisms per lung. The quantitation of cysts was done for all specimens because it is rapid and easy to interpret. In some instances, the homogenate was also stained with Diff-Quik (American Scientific Products, McGaw Park, Ill.), a variant of the Wright-Giemsa stain, for quantitation of the nuclei of all *P. carinii* developmental stages.

Histologic studies were performed on a selective basis to confirm the results of the quantitation of organisms. We have found that histologic examination correlates well with the quantitation of organisms but is less precise and more labor-intensive (9, 18). The left lung was inflated and fixed in formalin, sectioned, and stained with hematoxylin and eosin and methenamine silver; the intensity of the infection was graded from 0 to 4+ on the basis of the extent of alveolar involvement by an experienced pathologist. All specimens were read in a blinded manner.

Since the data did not follow a normal pattern of distribution and were frequently skewed, several different procedures were used for analysis (18, 19). Statistical techniques included the nonparametric Kruskal-Wallis test for the analysis of variance and the Wilcoxin rank sum test to compare individual groups with the Bonferroni correction for multiple comparisons; the overall $\alpha$ value was 0.05. The anti-*P. carinii* activity of the drugs tested was also classified by calculating the reduction in the median cyst or nucleus count in the treatment group compared with that in the C/S group in the same experiment. The results were expressed in the following categories: inactive, $\leq$5-fold reduction; slight activity, 5- to 9-fold reduction; moderate, 10- to 99-fold reduction; marked, 100- to 999-fold reduction; very marked activity, $\geq$1,000-fold reduction. Drugs were also classified as showing very marked activity in cases in which the median cyst count in the C/S group was $<10^9$ per lung and that of the treatment group was at the lower limit of detectability ($1.1 \times 10^5$ per lung).

RESULTS

SMX studies. SMX studies compared different dosages of DHFR inhibitors administered alone and in combination with a fixed low dosage (3 mg/kg/day) of SMX. Our previous report had established that this dosage reduced the median *P. carinii* cyst count by an amount which would enable us to detect the beneficial effects of the DHFR inhibitors in the regimen (18). The results shown in Fig. 2 and 3 are from the
same experiment. The analysis of variance in this study revealed a highly significant difference \( P < 0.0001 \) in the cyst counts among the different groups. The analysis by the Wilcoxon rank sum test showed that all treatment regimens except TMP and Ro 11-8958 used alone achieved significant \( P < 0.0001 \) reductions in cyst counts compared with those of the C/S group.

As seen in Fig. 2, SMX administered alone at a dosage of 3 mg/kg/day had moderate anti- \( P. carinii \) activity, reducing the median cyst count 14-fold from \( 7.1 \times 10^7 \) per lung in the control (C/S) group to \( 4.8 \times 10^6 \) per lung. By contrast, TMP used alone at a dosage of 100 mg/kg/day was ineffective. TMP administered at a dosage of 20 mg/kg/day combined with SMX reduced the median cyst count 59-fold to \( 1.2 \times 10^5 \) per lung, whereas TMP used at a dosage of 100 mg/kg/day with SMX showed marked activity, lowering the median cyst count 591-fold to \( 1.6 \times 10^5 \) per lung. These data were interpreted as indicating drug synergism, since the reduction in \( P. carinii \) cyst count with the TMP-SMX combination was greater than the sum of reductions achieved with TMP and SMX used alone. In fact, the results obtained with the TMP at 100 mg/kg/day and SMX regimen were comparable to those achieved with the standard regimen of TMP at 50 mg/kg/day and SMX at 250 mg/kg/day used as a positive control.

Ro 11-8958 used alone at a dosage of 100 mg/kg/day resulted in a median cyst count \( 4.8 \times 10^7 \) per lung which was very similar to that of the C/S group and thus was classified as inactive (Fig. 2). Ro 11-8958 administered at a dosage of 20 mg/kg/day in combination with SMX lowered the cyst count 11-fold to \( 6.4 \times 10^6 \) per lung. The higher (100 mg/kg/day) dosage combination of Ro 11-8958 and SMX reduced the cyst count 645-fold to \( 1.1 \times 10^5 \) per lung, the lower limit of detection. These data were similar to those achieved with TMP.

As shown in Fig. 3, a similar pattern of activity was also found with DIA. Our previous study showed that DIA administered alone at a dosage of 100 mg/kg/day was ineffective against \( P. carinii \) (21). In the present study, DIA used at dosages of 20 mg/kg/day and 100 mg/kg/day with SMX lowered the cyst counts to \( 2.0 \times 10^6 \) per lung and \( 1.1 \times 10^5 \) per lung, respectively. The regimens of DIA, TMP, and Ro 11-8958 combined with SMX were well tolerated by the rats.

PYR was administered alone at dosages of 3 and 6 mg/kg/day in combination with SMX and FA (Fig. 3). In earlier studies, we found that these dosages of PYR had little anti- \( P. carinii \) activity but were well tolerated when used with FA (18, 21). The 3- and 6-mg/kg/day regimens lowered the median cyst counts to \( 2.5 \times 10^6 \) per lung and \( 1.6 \times 10^5 \) per lung, respectively. These data suggested that PYR was less active than the other DHFR inhibitors against \( P. carinii \) at the dosages used.

The anti- \( P. carinii \) activities of some of the drugs used in this experiment were also compared by counting nuclei in order to analyze the effects of these agents on all developmental stages of the organism (Fig. 4). As in our previous
Although variance with DAP previous the results DAP reduced the lung cyst group to the same result in cysts which fell 4,545-fold to undetectable levels (1.1 × 10^5 per lung), indicating a synergistic effect.

DAP studies. Initial studies were patterned after our previous work with SMX, which was devoted to developing a dose-response curve for the drug (18). Administration of DAP at dosages of 5, 25, and 125 mg/kg/day resulted in median cyst counts of 3.2 × 10^6 per lung, 2.1 × 10^7 per lung, and 1.1 × 10^8 per lung, respectively, compared with a count of 7.1 × 10^7 per lung in the C/S group. The 25-mg/kg/day dosage was selected as the fixed low dosage of DAP with which to compare DHFR inhibitors.

All drugs were compared in the same experiment, for which the results are presented in Fig. 5 and 6. Analysis of variance revealed a highly significant difference (P < 0.0001) in cyst counts among the different rat groups. All treatment regimens resulted in significant (P < 0.004) reductions in the cyst counts compared with those of the C/S group. DAP administered alone at a dosage of 25 mg/kg/day lowered the median cyst count 8-fold from 5.0 × 10^6 per lung in the C/S group to 6.0 × 10^5 per lung. TMP at a dosage of 20 mg/kg/day with DAP lowered the cyst count slightly further to 2.8 × 10^5 per lung (Fig. 5). The regimen of TMP at 100 mg/kg/day and DAP reduced the cyst count 154-fold to 3.2 × 10^5 per lung. Although this drug combination was synergistic, it was not nearly as effective as the standard regimen of TMP at 50 mg/kg/day and SMX at 250 mg/kg/day, which lowered the cyst count 4,545-fold to undetectable levels (1.1 × 10^5 per lung).

Ro 11-8958 showed greater anti-P. carinii activity than TMP or the other DHFR inhibitors (Fig. 5). The regimen of Ro 11-8958 at 20 mg/kg/day with DAP lowered the median cyst count 142-fold to 3.5 × 10^5 per lung, whereas Ro 11-8958 administered at a dosage of 100 mg/kg/day with DAP reduced the cyst count 2,272-fold to 2.2 × 10^5 per lung.

DIA administered at dosages of 20 and 100 mg/kg/day with DAP lowered the median cyst count to 1.6 × 10^5 per lung and 2.3 × 10^5 per lung, respectively (Fig. 6); these data were similar to those achieved with TMP. PYR administered at a dosage of 3 mg/kg/day with DAP resulted in a cyst count of 4.3 × 10^5 per lung, which was very similar to that obtained with DAP alone (Fig. 6). The higher (6 mg/kg/day) PYR dosage in combination with DAP lowered the median cyst count 50-fold to 9.9 × 10^4 per lung. All regimens of DHFR inhibitors and DAP were well tolerated by the rats.

Additional studies. Further experiments analyzed the efficacy of additional dose regimens of Ro 11-8958 and other DHFR inhibitors. In one study, Ro 11-8958 administered at a dosage of 30 mg/kg/day with SMX lowered the median cyst count 53-fold from 1.4 × 10^6 per lung in the C/S group to 2.6 × 10^5 per lung. The regimen of Ro 11-8958 at 100 mg/kg/day with SMX showed very marked activity, lowering the cyst count 1,272-fold to 1.1 × 10^5 per lung.

The same experiment investigated the DHFR inhibitor cycloguanil, which was developed as an antimalarial drug. Preliminary data indicated that this agent used alone at a dosage of 20 mg/kg/day intramuscularly (the maximal dosage tolerated by the immunosuppressed rats) was ineffective against P. carinii. Administration of cycloguanil at 20 mg/kg/day with SMX lowered the median cyst count 10-fold to 1.4 × 10^5 per lung, a reduction very similar to that achieved with SMX alone (median cyst count of 1.1 × 10^5 per lung).

A small part of the DAP studies described above was also devoted to analyzing the anti-P. carinii activity of tetrox-
oprim, a DHFR inhibitor structurally related to TMP which had been occasionally used in the rat model of pneumocystosis (8). Since limited quantities of tetroxoprim were available, the drug was tested at only one dosage alone and in combination with SMX. Administration of tetroxoprim alone at 50 mg/kg/day lowered the median cyst count eightfold from $5.0 \times 10^5$ per lung in the C/S group to $6.0 \times 10^7$ per lung; the addition of SMX to the regimen lowered the median cyst count to only $3.3 \times 10^7$ per lung.

**Histologic analysis.** Evaluation of the magnitude of *P. carinii* pneumonia and the effects of the antimicrobial drugs by the histologic scoring system correlated well with the results obtained by the quantitation of organisms. Typical features of the disease include a foamy, honeycombed alveolar exudate and a minimal host inflammatory response on hematoxylin and eosin staining and clusters of organisms in the alveoli on methenamine silver staining.

**DISCUSSION**

Although the immunosuppressed rat model has been a valuable tool for anti-*P. carinii* drug development, it has been of little help in studying the synergistic effects of antifolate compounds. TMP, the prototype DHFR inhibitor, has been uniformly ineffective against *P. carinii* when administered alone in this model (7, 11, 21). Proposed reasons for this lack of efficacy have ranged from high levels of thymidine in serum in rats to the short half-life of TMP or other factors related to its metabolism (18). SMX, the prototype sulfonamide, is so active against the organism that partial efficacy could be achieved with only low dosages (e.g., 3 mg/kg/day) of the drug; yet, even here, the addition of TMP at 0.6 mg/kg/day (the standard TMP-SMX ratio of 1:5) did not improve the effectiveness of SMX (18).

In the present study, we reversed the TMP-SMX ratio by using high dosages of TMP in combination with a fixed, low dosage of SMX. This approach allowed DHFR inhibitors to be compared directly under the same experimental conditions. Drug synergism developed in a dose-dependent manner and was very noticeable at a TMP dosage of 100 mg/kg/day. When analyzed in the same experiment, TMP, Ro 11-8958, and DIA exhibited comparable anti-*P. carinii* activity. By contrast, little synergy was found with PYR, cycloguanil, and tetroxoprim; however, evaluation of these agents was hampered by dose-limiting toxicity or small quantities of the drug.

The same strategy used with SMX was applied to DAP. While drug synergism was found with TMP and DIA, the magnitude of the *P. carinii* cyst reduction was considerably less than that found when TMP and DIA were combined with SMX. By contrast, the regimen of Ro-8958-DAP and Ro 11-8958-SMX exhibited similar anti-*P. carinii* activity. These data, together with the SMX results described above, illustrate some important points in attempting to find optimal

**FIG. 5.** Treatment of pneumocystosis with TMP, Ro 11-8958, and DAP as assessed by the number of cysts per lung. The DAP dosage was 25 mg/kg/day p.o. throughout. The dosage (indicated in parentheses in the figure) regimens included TMP, 20 mg/kg/day p.o. with DAP; TMP, 100 mg/kg/day p.o. with DAP; Ro 11-8958, 20 mg/kg/day with DAP; Ro 11-8958, 100 mg/kg/day with DAP; and DAP. C/S, controls receiving steroids; TMP(50)-SMX(250), TMP at 50 mg/kg/day p.o. and SMX at 250 mg/kg/day p.o. (another control group receiving the standard treatment regimen). Horizontal bars indicate median values.
combinations of antifolate drugs in the rat model of pneumocystosis. First, it is necessary to compare different DHFR inhibitors, sulfonamides, and sulfones because some combinations may be more active than others. Second, more than one dosage of these agents must be used to ensure that synergistic effects are not overlooked.

Ro 11-8958 is one of several trimethoprim analogs which contain alterations in the benzyl moiety (16). Ro 11-8958's attractive features include enhanced activity against gram-positive cocci, nocardia, and anaerobes; a larger volume of distribution; and a longer half-life. Ro 11-8958 is about 10 times more active than TMP against rat P. carinii DHFR in vitro; this enhanced effect is also more favorable in terms of specificity (i.e., compared with activity against rat or human DHFR) (16a). These characteristics, combined with the greater activity of the Ro 11-8958–DAP regimen, point to Ro 11-8958 as a favorable candidate for clinical trials.

However, the development of new DHFR inhibitors as antimicrobial agents for use in humans poses several potential problems. One factor to be considered is the possibility that superior activity in vitro does not translate into better activity in vivo. For example, trimetrexate, which has greater activity than TMP against P. carinii DHFR in vitro (2), was limited by problems of efficacy and toxicity when tested in patients with pneumocystosis (1). Another issue to be decided is whether to attempt to use the new DHFR inhibitor in humans alone or in combination with a sulfonamide or sulfone, as is the current clinical practice. Sulfonamides and sulfones cause frequent adverse reactions in human immunodeficiency virus patients, and this problem would probably not be influenced by the presence of a new DHFR inhibitor. Yet, recent success in cloning the P. carinii gene encoding dihydropteroate synthase, the target of sulfonamides, offers hope that new inhibitors which are less toxic can be developed (17).

In summary, we have developed a new method of testing antifolate drugs in the immunosuppressed rat model of pneumocystosis which allows different dosages of DHFR inhibitors to be studied in combination with a fixed, low dosage of a sulfonamide or sulfone. This system will be helpful in developing new agents as well as in studying their structure-activity relationships and drug interactions.

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