Lepr. Rev. (1970) **41**, 223-228

The Minimal Inhibitory Concentrations of Sulphadimethozine and Sulphadoxine Against *Mycobacterium leprae*[*]

G. A. ELLARD, PATRICIA T. GAMMON

Medical Research Council's Unit for Laboratory Studies of Tuberculosis, Royal Postgraduate Medical School, London, W.12

R. J. W. REES

Laboratory for Leprosy and Mycobacterial Research†, National Institute for Medical Research, Mill Hill, London, N.W.7

Sulphadimethoxine inhibited the multiplication of *Myco. leprae* in the mouse footpad system when given at a dietary concentration of 0.01%, but not at one of 0.001%. Sulphadoxine inhibited the multiplication of *Myco. leprae* at a dietary concentration of 0.04%, but not at one of 0.004%. Determination of the plasma concentrations of the 2 drugs in mice fed with these dietary concentrations of sulphadimethoxine and sulphadoxine indicated that the MICs of these 2 compounds against *Myco. leprae* were between 20 and 35 μg per ml. The significance of these findings is discussed, both in relation to the previously determined MIC of dapsone against *Myco. leprae* and to the use of these 2 long-acting sulphonamides in the treatment of human leprosy.

Sulphadimethoxine (2,6-dimethoxy-4-sulphanilamido-pyrimidine) and sulphadoxine (5,6-dimethoxy-4-sulphanilamido-pyrimidine) are 2 long-acting sulphonamides that have been shown to prevent the multiplication of *Mycobacterium leprae* in the mouse footpad test (Rees, 1965; Gaugas, 1967). Both compounds have been used in the treatment of human leprosy (Opromolla, 1962; Languillon, 1964; Currie, 1966; Gaind *et al.*, 1966; Languillon, 1969). Since *Myco. leprae* cannot be cultivated *in vitro*, the minimal inhibitory concentration (MIC) of an anti-leprosy drug can only be established *in vivo* by determining its concentration in the serum or plasma of mice fed with the minimum dose of the compound that effectively inhibits the multiplication of *Myco. leprae* in the mouse footpad. So far these combined techniques have been used to determine the MIC of only one anti-leprosy drug, 4,4[1]-diaminodiphenyl-sulphone (dapsone, DDS) (Shepard *et al.*, 1966; Rees, 1967*a*). In this paper we describe the methods used and the results obtained in determining the MICs of sulphadimethoxine and of sulphadoxine.

*Received for publication May, 1970.

†This laboratory is designated a WHO Regional Reference Centre for *Mycobacterium leprae*.

DETERMINATION OF THE MINIMUM DOSES OF SULPHADIMETHOXINE AND SULPHADOXINE REQUIRED TO INHIBIT THE MULTIPLICATION OF *MYCO. LEPRAE*

The methods used for infecting and determining the multiplication of *Myco. leprae* in the footpads of mice were based on those previously described (Rees, 1965) and subsequently modified for chemotherapeutic studies (Rees, 1967*b*). Briefly, the hind footpads of female P-strain albino mice, in groups of 6, were inoculated with 10^4 acid-fast bacilli derived from homogenates of skin nodules from previously untreated patients with active lepromatous leprosy. In the present studies, 2 strains of *Myco. leprae* from previously untreated patients were used. One of these

strains was also shown to be sensitive to inhibition by 0.0001% dapsone in the diet, demonstrating directly that its sensitivity to dapsone was similar to that of all the other strains of *Myco. leprae* isolated from previously untreated patients (Shepard, 1967; Rees, 1967*a*; Shepard *et al.*, 1969). This standard procedure would be expected to result in the multiplication of *Myco. leprae*, yielding a 100-fold increase in acid-fast bacilli 6 months later in untreated animals. Such multiplication was confirmed by determining the number of acid-fast bacilli in the homogenates of both individual hind footpads from one of the mice in the untreated group killed 6 months after infection. With this confirmation, all the mice from both the untreated and treated groups of animals were killed within the next 2 months, and the number of acid-fast bacilli determined in homogenates from individual hind footpads from all the animals. The quantitative bacteriological methods used in these experiments registered a minimum count of 5×10^4 acid-fast bacilli per footpad homogenate.

Treatment was started on the day of infection and continued throughout the experiment by providing 5 g per mouse per day of powdered 41B diet (Bruce, 1950). The drugs in powder form were incorporated in the diet by thorough mixing in a mechanical food-mixer. Previous data (Rees, 1965) had shown that sulphadimethoxine and sulphadoxine inhibited the multiplication of *Myco. leprae* in the mouse footpad test at 0.1 and 0.04% of the drug in the diet, respectively. Therefore, in the present studies designed to determine the minimum doses of these compounds that would inhibit the multiplication of *Myco. leprae*, these 2 doses together with doses one-tenth and one-hundredth of the above were used. Other groups of 6 mice were fed for 14 days with either 0.001% or 0.01% or 0.1% sulphadimethoxine or 0.004% or 0.04% sulphadoxine in the diet, and the plasma concentrations of the sulphonamides determined in individual mice by the fluorometric and colorimetric methods described below.

DETERMINATION OF THE CONCENTRATION OF SULPHADIMETHOXINE AND SULPHADOXINE IN MOUSE PLASMA

Standards consisted of normal mouse plasma to which sulphadimethoxine or sulphadoxine had been added to a concentration of 75 μg per ml. Solvents and reagents were of analytical grade and the ethyl acetate was redistilled before use. Aliquots of mouse plasma (0.2 ml) were pipetted into small stoppered centrifuge tubes together with 2.8 ml of water and 1.0 ml of M/15 pH 7.0 phosphate buffer, and extracted by shaking with 6.0 ml of ethyl acetate. The phases were separated by centrifugation, and the ethyl acetate extract decanted, using a Pasteur pipette, and dried by shaking with 0.5 g of anhydrous sodium sulphate. Sulphadimethoxine was then determined fluorimetrically by measuring the fluorescence of the dried ethyl acetate extract in an Aminco-Bowman spectrophotofluorometer at 332 mμ, the excitation wavelength being 280 mμ. In a similar way, sulphadoxine was determined by measuring the fluorescence of the ethyl acetate extract at 288/330 mμ. Then 3 ml of the ethyl acetate extract was extracted by shaking with 1 ml of 2 N hydrochloric acid, and the sulphadimethoxine and sulphadoxine determined colorimetrically in the 2 N hydrochloric acid extract by a modification of the procedure of Bratton and Marshall (1939). In this modification 0.3 ml of ethanol was added to 0.3 ml of the 2 N hydrochloric acid extract and reacted with 0.01 ml of 1% (w/v) aqueous sodium nitrite. After 5 min the nitrite was destroyed by the addition of 0.01 ml of 10% (w/v) aqueous ammonium sulphamate; after another 5 min 0.01 ml of 2% (w/v) N-1-naphthyl-ethylene-diamine-dihydrochloride was added in acetone/water (1 : 1 by volume) and the optical density measured at 545 mμ after a further 15 min.

RESULTS AND DISCUSSION

The results are summarized in Table I and illustrated in Fig. 1. Little diurnal variation in the plasma concentrations of these compounds was expected, in view of the fact that Böhni and

TABLE 1

Concentrations of sulphadimethoxine and sulphadoxine in mouse plasma and their inhibition of the multiplication of *Myco. leprae*

Drug	*Dose* (% *in diet*)	*Activity*	*Concentration in mouse plasma* (μg/ml) *Fluorimetric method*	*Colorimetric method*
Sulphadimethoxine	0.001	0	12.4±3.4*	7.7±3.0
	0.01	+	37.1±5.1	30.6±6.7
	0.1	+	77.5±11.6	73.9±11.6
Sulphadoxine	0.0004	0	———†	———
	0.004	0	22.3±8.8	20.2±2.7
	0.04	+	62.1±8.9	60.9±11.3

+ = full activity. 0 = inactive. * Mean ± S.D. observations. † Not measured.

her collaborators (1969) found the half-lives of sulphadimethoxine and sulphadoxine in the mouse to be 28 and 38 hr, respectively. Furthermore, after 14 days' continuous feeding in the diet, both the drugs should have reached equilibrium concentrations.

The principal metabolites of these sulphonamides, their N^4-acetyl derivatives and glucuronide conjugates, are devoid of antimicrobial activity (for a recent review, see Böhni *et al.*, 1969). It was anticipated that these metabolites would not be determined by either the fluorometric or the colorimetric methods, since the glucuronides would not extract into ethyl acetate at pH 7.0 and the N^4-acetyl derivatives would not be expected to fluoresce, and cannot be diazotized and coupled by the Bratton and Marshall procedure. The fact that similar results were obtained using both the fluorometric and colorimetric methods supports the conclusion that both methods specifically measured the microbiologically-active unchanged sulphonamides.

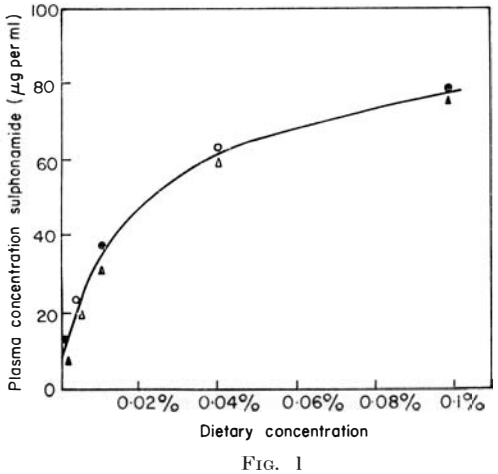

FIG. 1

● Sulphadimethoxine (fluorometric method).
▲ Sulphadimethoxine (colorimetric method).
○ Sulphadoxine (fluorometric method).
△ Sulphadoxine (colorimetric method).

It will be noted that, with both drugs, the plasma concentrations did not increase proportionally to the amount of drug given in the diet. Although it is conceivable that the absorption of sulphadimethoxine and sulphadoxine is less complete at the higher dietary concentrations, Böhni *et al.* (1969) had previously obtained evidence that when sulphadoxine is administered orally to the mouse in therapeutic doses it is almost completely absorbed. A more likely explanation of our results is that the half-life of these 2 sulphonamides decreases with increasing size of dose. Thus, while sulphadoxine has a half-life of 38 hr in mice when given at a dose of 8.7 mg per kg body-weight (Rieder and Böhni, 1964), after a dose of 200 mg per kg its half-life is only 20 hr (Rieder and Böhni, personal communication). The fact that the data for both sulphadimethoxine and sulphamethoxine can be conveniently plotted on the same curve (see Fig. 1) suggests that equal doses of these drugs would give rise to very similar plasma concentrations in the mouse.

Since the multiplication of *Myco. leprae* in the mouse footpad is inhibited by a dietary concentration of 0.01% sulphadimethoxine but

not by a concentration of 0.001%, and since these dietary concentrations result in plasma concentrations of 34 μg per ml and 10 μg per ml of the drug respectively, it was concluded that the MIC of sulphadimethoxine against *Myco. leprae* is about 20 μg per ml. Similarly the multiplication of *Myco. leprae* was inhibited by sulphadoxine in a plasma concentration of 61 μg per ml, but not by one of 21 μg per ml. The MIC of sulphadoxine against *Myco. leprae* is therefore about 35 μg per ml. However, since the dietary concentrations used for testing sulphadimethoxine and sulphadoxine were widely separated, it cannot be inferred that the MICs of these 2 compounds against *Myco. leprae* differ significantly.

The average weight of the mice was 25 g. Dosage with 0.01% (active) and 0.004% (inactive) of these drugs was therefore equivalent to daily dosage with 20 and 8 mg per kg body weight, respectively, of these sulphonamides. The 50% curative dose (CD_{50}) of these compounds against *Myco. leprae* in the mouse must therefore lie between these 2 doses. The results reported by Gaugas (1967), who found that the multiplication of *Myco. leprae* in the mouse footpad was completely inhibited by daily oral administration of single doses equivalent to 75 mg per kg body weight (sulphadimethoxine) and 25 mg per kg body weight (sulphadoxine), are in accord with these conclusions. These CD_{50}s are similar to that obtained by Böhni *et al.* (1969) for sulphadimethoxine against infections of *Escherichia coli* in the mouse, and are from 2 to 20 times greater than the CD_{50}s obtained for both compounds against infections with *Staphylococcus aureus*, *Streptococcus haemolyticus*, *Proteus vulgaris* and *Klebsiella pneumoniae*.

DISCUSSION

These results may be contrasted with those obtained with dapsone, which consistently inhibits the multiplication in the mouse footpad of strains of *Myco. leprae* derived from untreated patients, at a dietary concentration as low as 0.0001% (Shepard *et al.*, 1966, 1969; Shepard, 1967; Rees, 1967*a*, *b*). Estimation of the serum or plasma concentrations of dapsone in mice fed with 0.001 to 0.1% of the sulphone in the diet, indicated that a dietary concentration of 0.0001% would give rise to plasma concentrations of the drug of only about 0.01 to 0.015 μg per ml (Shepard *et al.*, 1966; Rees, 1967*a*; Ellard *et al.*, 1971). Thus, it would appear that while *Myco. leprae* is exquisitely sensitive to dapsone, it is some 2000 times less sensitive to sulphadimethoxine and sulphadoxine.

The MIC of dapsone against *Myco. leprae*, as determined in the mouse footpad system in this way, not only explains the effectiveness of standard doses of 100 to 600 mg of dapsone per week in the treatment of human leprosy, but has been used to predict the efficacy of doses of as little as 1 mg of dapsone per day (Waters and Rees, 1971). It is pertinent, therefore, to consider the clinical implications of these determinations of the MIC of sulphadimethoxine and of sulphadoxine against *Myco. leprae*.

When used for the treatment of human leprosy, sulphadimethoxine is normally given in doses of either 0.75 g on alternate days (Languillon, 1964) or 1.5 g daily (Opromolla, 1962). The half-life of sulphadimethoxine in man is about 36 hr (Brandman *et al.*, 1959; Madsen and Iversen, 1964). Consequently, daily administration of the drug results in a progressive rise in plasma concentrations until after about a week a plateau is reached. Madsen (1961) found that the plasma concentration of sulphadimethoxine immediately before daily dosage with 0.5 g of the drug rose to about 60 μg per ml after 5 days and remained constant thereafter. Brandman *et al.* (1959) found peak blood concentrations of about 60 μg per ml 4 hr after dosage with 1 g of the drug, and after repeated daily dosage a plateau of about 70 μg per ml immediately before the next daily dose. Assuming that sulphadimethoxine, like sulphadoxine, does not penetrate into the blood red cells to any significant extent (Böhni *et al.*, 1969), such a plateau blood concentration would be equivalent to a plasma concentration of about 120 μg per ml. It would therefore appear that

repeated daily dosage with 1.5 g of sulphadimethoxine would lead to the attainment of plasma concentrations fluctuating between about 180 and 300 μg per ml.

Sulphadoxine is usually given in a dosage of 1 to 1.5 g per week when used for the treatment of human leprosy (Currie, 1966; Gaind *et al.*, 1966; Languillon, 1969). Its half-life in man is about one week (Portwich and Büttner, 1964; Böhni *et al.*, 1969), and repeated daily dosage with 1 g of sulphadoxine eventually gives plasma concentrations that fluctuate between about 100 and 200 μg per ml (Böhni *et al.*, 1969).

Thus the plasma concentrations of these 2 sulphonamides that are achieved in man at the dosages usually given in the treatment of leprosy range from about 150 to 300 μg per ml. This is between 4 and 15 times their MICs against *Myco. leprae* as determined in the mouse footpad test. It is therefore suggested that the treatment of human leprosy with sulphadimethoxine and sulphadoxine is comparable to the use of dapsone in doses of less than 15 mg per day or of giving injections of 225 mg 4,4[1] diacetyl diamino diphenyl sulphone (DADDS) every 11 weeks (Shepard *et al.*, 1968). Great care should therefore be taken to establish whether or not relapse can occur during treatment with these 2 long-acting sulphonamides because of the appearance of drug-resistant mutants of *Myco. leprae* (Rees, 1967*a*, *b*; Shepard *et al.*, 1969). Since cross-resistance has been demonstrated in one strain of *Myco. leprae* between dapsone and sulphadimethoxine (Adams and Waters, 1966) and between dapsone, sulphadimethoxine and sulphadoxine in 4 other strains of this organism (Rees, 1967*a*) patients who relapse during treatment with sulphadimethoxine or sulphadoxine, owing to the emergence of drug resistance, may fail to respond to subsequent treatment with dapsone.

ACKNOWLEDGEMENT

We are grateful to Hoffman-La Roche and Co. for the supplies of sulphadimethoxine and sulphadoxine used in these studies. These were obtained through the courtesy of Dr. J. M. B. Garrod, whom we should also like to thank for providing us with literature on the 2 drugs.

REFERENCES

ADAMS, A. R. D. and WATERS, M. F. R. (1966). Dapsone-resistant lepromatous leprosy in England. *Br. med. J. ii*, 872.

BÖHNI, E., FUST, B., RIEDER, J., SCHAERER, K. and HAVAS, L. (1969). Comparative toxicological, chemotherapeutic and pharmacokinetic studies with sulphormethoxine and other sulphonamides in animals and man. *Chemotherapy* **14**, 195.

BRANDMAN, O., OYER, C. and ENGLEBERG, R. (1959). Blood levels and urinary excretion of a long-acting sulphonamide. *J. med. Soc. N.J.* **56**, 24.

BRATTON, A. C. and MARSHALL, E. K., JR. (1939). A new coupling component for sulfanilamide determination. *J. biol. Chem.* **128**, 537.

BRUCE, H. M. (1950). Feeding and breeding of laboratory animals, XI. *J. Hyg., Camb.* **48**, 171.

CURRIE, G. (1966). A clinical trial of sulforthomidine ("Fanasil" Roche) in lepromatous leprosy. *Lepr. Rev.* **37**, 205.

ELLARD, G. A., GAMMON, P. T., REES, R. J. W. and WATERS, M. F. R. (1971). Studies on the determination of the minimal inhibitory concentration (MIC) of 4,4[1]-diamino-diphenyl-sulphone (DDS, dapsone) against *Mycobacterium leprae*. (In preparation.)

GAIND, M. L., MENOR, C. V. and RAMAKRISHNA (1966). Therapeutic trial report on long-acting sulphonamide Ro 4-4393 (Fanasil) in the treatment of leprosy. *Lepr. Rev.* **37**, 167.

GAUGAS, J. M. (1967). Antimicrobial therapy of experimental human leprosy (*Myco. leprae*) infection in the mouse foot-pad. *Lepr. Rev.* **38**, 225.

LANGUILLON, J. (1964). La sulfamidothérapie dans la lèpre (sulfaméthoxypyridazine, acétylsulfaméthoxypyridazine, sulfadiméthoxine, acétylsulfaméthoxypyrazine, Ro 4-4393). *Méd. trop. Marseilles* **24**, 522.

LANGUILLON, J. (1969). Results of specific treatment of leprosy with long-acting sulphonamides. *Int. J. Lepr.* **36**, 654.

MADSEN, S. T. (1961). Plasma concentration and renal excretion of sulfadimethoxine during long-term treatment. *Antibiot. Med. clin. Ther.* **8**, 87.

MADSEN, S. T. and IVERSEN, P. F. (1964). Metabolic problems during treatment with long-acting sulfonamides. In *3rd Int. Congr. on Chemotherapy, Stuttgart* 1963, **1**, 644. Thieme, Stuttgart.

OPROMOLLA, D. V. A. (1962). A sulfametoxina no tratamento da lepra. *Revta. Bras. Leprol.* **30**, 21.

PORTWICH, F. and BÜTTNER, H. (1964). Zur Pharmakokinetik eines langwirkenden Sulfonamids (4-Sulfanilamido-5, 6-dimethoxypyrimidin) beim gesunden Menschen. *Klin. Wschr.* **42**, 740.

REES, R. J. W. (1964). Limited multiplication of acid-fast bacilli in the foot-pads of mice inoculated with *Mycobacterium leprae*. *Br. J. exp. Path.* **45**, 207.

REES, R. J. W. (1965). Recent bacteriologic, immunologic and pathologic studies on experimental human leprosy in the mouse foot-pad. *Int. J. Lepr.* **33**, 646.

REES, R. J. W. (1967*a*). Drug resistance of *Mycobacterium leprae* particularly to DDS. *Int. J. Lepr.* **35**, 625.

REES, R. J. W. (1967*b*). Leprosy. A preliminary review of the experimental evaluation of drugs for the treatment of leprosy. *Trans. R. Soc. Trop. Med. Hyg.* **61**, 581.

REES, R. J. W. (1969). New prospects for the study of leprosy in the laboratory. *Bull. Wld Hlth Org.* **40**, 785.

RIEDER, J. and BÖHNI, E. (1964). Vergleichende Untersuchungen über die chemotherapeutisch wirksamen Konzentrationen von Sulfonamiden bei künstlich infizierten Versuchstieren. In *3rd Int. Congr. on Chemotherapy, Stuttgart* 1963, **1**, 622. Thieme, Stuttgart.

SHEPARD, C. C. (1967). Studies in mice of the action of DDS against *Mycobacterium leprae*. *Int. J. Lepr.* **35**, 616.

SHEPARD, C. C., LEVY, L. and FASAL, P. (1969). The sensitivity to dapsone (DDS) of *Mycobacterium leprae* from patients with and without previous treatment. *Am. J. trop. Med. Hyg.* **18**, 258.

SHEPARD, C. C., MCRAE, D. H. and HABAS, J. A. (1966). Sensitivity of *Mycobacterium leprae* to low levels of 4,4^1-diaminodiphenyl sulphone. *Proc. Soc. exp. Biol. Med.* **122**, 893.

SHEPARD, C. C., TOLENTINO, J. G. and MCRAE, D. H. (1968). The therapeutic effect of 4,4^1-diacetyldiamino-diphenylsulfone (DADDS) in leprosy. *Am. J. trop. Med. Hyg.* **17**, 192.

WATERS, M. F. R. and REES, R. J. W. (1971). Pilot trial of dapsone, 1 mg daily, in the treatment of lepromatous leprosy. (In preparation.)