CROSS RESISTANCE OF PYRIMETHAMINE AND SULFADOXINE TO THEIR RELATED COMPOUNDS IN *PLASMODIUM FALCIPARUM*

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INTRODUCTION

The pyrimethamine resistance in *Plasmodium falciparum* developed soon after this compound began to be used (Mcgregor and Smith, 1952). In view of structural resemblance between pyrimethamine and other drugs in the same group cross resistance might be possible. The observations by Clyde (1960) and Contacos *et al.*, (1965) in patients infected with *P. falciparum* suggested that there might be a cross resistance of pyrimethamine to cycloguanil and proguanil. If this aspect does really occur in nature it would certainly cause a problem in introducing a new substance to replace pyrimethamine which is now still used for prophylaxis of human malaria, particularly where *P. vivax* and *P. malariae* predominate. In addition, pyrimethamine is also used to combine with sulfadoxine for treatment of chloroquine resistant *P. falciparum* which is widespread in Southeast Asia (Bruce-Chwatt, 1981).

Although drug resistance is generally specific for particular drugs it has been found to cross to either chemically related or unrelated compounds (Peters, 1970). The aim of this study is to investigate the possibility of pyrimethamine to cross resist with the drug such as amethopterin which share the same mode of action namely inhibiting the enzyme dihydrofolate reductase but this drug has never been used for malarial treatment before. The cross resistance between sulfadoxine and the other sulfonamides is studied due to its wide use in a combination with pyrimethamine which is commonly known as Fansidar^{R} (Roche).

MATERIALS AND METHODS

Parasites: Four culture lines of *Plasmodium falciparum* were used in this study. Line FCK was isolated in January 1979 from a Thai patient who visited the malaria clinic in Kanchanaburi, Western Thailand. Line T-17 was isolated from a Burmese patient in Tak in March 1981. The Gambian strain, line GS, was isolated from a patient in the Gambia and cultured continuously since 1979. This line was sensitive to pyrimethamine. GR originated from the same isolate as GS, but was later produced to become resistant to pyrimethamine (Tan-ariya, 1982). All four lines have been kept in continuous culture in type O human erythrocytes in a Petri dish and candle jar system, as described by Trager and Jensen (1976).

Culture media: The culture media used for continuous cultivation was RPMI 1640, with L-glutamine and without bicarbonate (Grand Island Biological Company, Grand Island, N.Y.). This medium was reconstituted as described by Trager and Jensen (1976). For sensitivity test, Waymouth powder, MB 752/1, was used in place of RPMI because Waymouth contained no p-amino benzoic acid, a growth factor which has been shown to give erroneous results in the *in vitro* tests of *P. falciparum* sensitivity to sulfa drugs (Tan-ariya, 1982). The preparation of Way-

mouth was the same as previously described (Brockelman and Tan-ariya, 1982).

Drugs: Pyrimethamine and sulfadoxine used in this study were obtained upon special request from F. Hoffmann-La Roche Laboratory, Basle, Switzerland as sterile stock solutions of 0.01 M and 1.0 M concentration, respectively. Sulfisoxasole^R or sulfasol^R (2 g/5 ml), Pliva Pharmaceutical and Chemical Works, Zegreb, Yugoslavia and sulfadiazine sodium (1 g/4 ml), May and Baker Ltd., Dagenham, England were purchased from a local drugstore as sterile aqueous solutions in ampoules for parenteral administration. A 1 mM stock solution of amethopterin was prepared by dissolving 0.00454 g of powder in 10 ml of 0.01 N NaOH and sterilized by filtration.

Stock solution of all drugs were diluted 100-fold with Waymouth to working solutions. These working solutions were then diluted with complete Waymouth medium containing 7% human serum to the final desired concentrations.

Experiments: The assay for drug sensitivity followed the vial-candle jar test described by Brockelman and Tan-ariya (1982). Briefly, parasites were grown in RPMI 1640 in 60-mm Petri dishes for 4-6 days. When parasitaemia was 10%, the cultures were pooled and washed repeatedly with complete Waymouth medium. The stock culture was diluted with freshly washed erythrocytes and suspended in an equal volume of Waymouth to result in an initial parasitaemia of 0.5-1%. This working culture was finally diluted to 10% erythrocyte suspension and distributed into small vials (2 × 3 cm). After the erythrocytes have settled the supernatants were replaced by Waymouth medium containing drugs. For each experiment, at least four concentrations, with four replicates for each drug concentration were tested. The cultures were exposed to the drugs for 48 hours without changing medium at 38°C in a candle jar.

Evaluation: Giemsa-stained thin blood smears were made before and after drug treatment. Parasite responses to drugs were evaluated by counting the number of parasites per 10,000 erythrocytes. Using these data, the common logarithm of the multplication factor, defined as the parasite multiplication rate (PMR), was calculated (Brockelman *et al.*, 1981). A drug concentration which allowed a treated parasites populaton to multiply to only one half of the control was regarded as the minimal inhibitory concentration for 50% suppression (MIC_{50}).

RESULTS

Cross resistance of pyrimethamine to amethopterin: The study was undertaken using both pyrimethamine sensitive (GS) and pyrimethamine resistant parasites (GR and T-17). The responses of the three isolates to amethopterin at varying concentrations are shown in Fig. 1. The results show that only 0.008 nmole of amethopterin per ml was

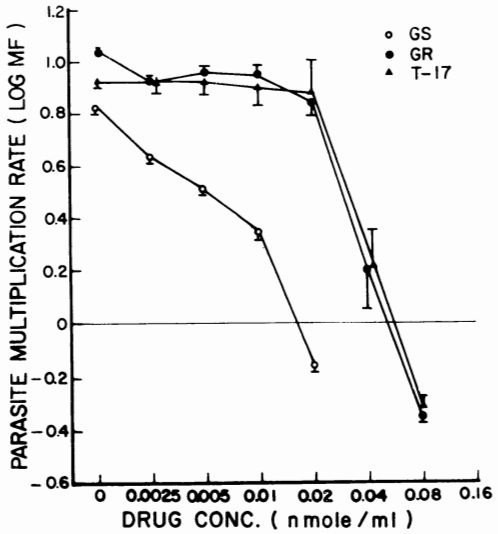

Fig. 1—Responses of the pyrimethamine sensitive line (GS) and the pyrimethamine resistant lines (GR and T-17) of *Plasmodium falciparum* to amethopterin. Each point represents mean PMR of four replicates. Vertical lines represent standard deviation of the means.

Table 1

The minimal inhibitory concentration of amethopterin required to produce 50% growth inhibition in Waymouth medium of the pyrimethamine sensitive line (GS) and the pyrimethamine resistant lines (GR and T-17) of *Plasmodium falciparum*.

Culture lines	Pyrimethamine (nmole/ml)		Amethopterin (nmole/ml)	
	MIC_{50}	Complete inhibition conc.	MIC_{50}	Complete inhibition conc.
GS	0.0025	0.0250	0.008	0.025
T-17	5.00	10.00	0.031	0.10
GR	5.00	10.00	0.031	0.10

enough to inhibit 50% of parasite growth in GS culture. Complete inhibition occurred at 0.025 nmole/ml. By contrast, the MIC_{50} of GR and T-17 (Table 1) which were pyrimethamine resistant, were 0.031 nmole/ml; for complete growth inhibition 0.10 nmole/ml was required. This indicates that the pyrimethamine resistant lines required 3.9 times (0.031 : 0.008) higher drug concentration than the sensitive one for 50% inhibition.

Cross resistance among sulfonamides: In order to determine whether there will be a cross resistance among the drugs in sulfonamide group, sulfisoxasole and sulfadiazine were chosen for the study. The sulfadoxine resistant parasites used were lines GS, GR, T-17 and FCK. The MIC_{50} of three sulfonamides for four lines are shown in Table 2. The GS and FCK which showed a similarity in sulfadoxine response were inhibited by different concentrations of sulfadiazine, namely 3000 for GS and 800 nmole/ml for FCK. The least sulfadoxine resistant line, i.e., T-17 was inhibited by sulfadiazine at 1000 nmole/ml which was higher than that dose required for the more resistant FCK. The most resistant sulfadoxine line, GR, was

Table 2

The minimal inhibitory concentrations of sulfadoxine, sulfisoxasole, and sulfadiazine required to produce 50% growth inhibition of various isolates of *Plasmodium falciparum* in Waymouth medium.

Culture lines	Minimal inhibitory concentrations (nmole/ml)		
	Sulfadoxine	Sulfadiazine	Sulfisoxasole
GS	100	3000	400
GR	200	2000	400
FCK	100	800	400
T-17	50	1000	400

inhibited by sulfadiazine at the concentration of 2000 nmole/ml which was not the highest concentration of sulfadiazine required among four lines.

Two isolates which had the same inhibitory concentration of sulfadoxine were inhibited by the same concentration of sulfisoxasole. GS and FCK lines which responded to 100 nmole of sulfadoxine per ml were found to be inhibited by sulfisoxasole at concentration of 400 nmole/ml. In addition, T-17 and GR which were resistant to sulfadoxine at 50 and 200 nmole/ml respectively were also inhibited by the same sulfisoxasole level (400 nmole/ml). The results indicate no difference in drug sensitivity of the four isolates used regardless of their differences in sulfadoxine sensitivity. It also shows that 50% inhibition was first achieved after the concentrations of sulfisoxasole was raised to be higher than sulfadoxine by 2 to 8 times. Only 50-200 nmole/ml of sulfadoxine was sufficient for 50% inhibition but a concentration as high as 400 nmole/ml of sulfisoxasole was required to reach the same inhibitory level.

The inhibitory concentrations of sulfisoxasole was 2.0 to 7.5 times lower than sulfadia-

zine which indicate a more effective activity of sulfisoxasole. Despite its higher activity, sulfisoxasole could not distinguish the difference in sulfonamide sensitivity of various *P. falciparum* isolates as clearly pronounced by sulfadiazine.

DISCUSSION

Amethopterin, an antileukemic agent structurally analogous to dihydrofolate, was shown to have some relation to pyrimethamine in this study. It can be seen from the results that pyrimethamine sensitive parasites were inhibited by a lower concentration of amethopterin than were resistant parasites. Moreover, two resistant strains which showed the same level of response to pyrimethamine were inhibited by amethopterin at the same concentration. The observation that the inhibitory concentration of amethopterin was lower than pyrimethamine could be explained by a difference in the Ki (Inhibitory dissociation constants for compounds combining with dihydrofolate reductase) between the two drugs. The Ki of amethopterin is lower than that of pyrimethamine, i.e., 9.4×10^{-12} M and 4.4×10^{-9} M respectively (Wang and Werkheiser, 1964). The comparative inhibition of the two drugs on growth of the bacterial strain, *Pediococcus cerevisae*, revealed that only 14×10^8 M/kg cells of amethopterin was enough to show 50% inhibition whereas $5{,}470 \times 10^8$ M/kg cells of pyrimethamine was needed (Nichol, 1959). The results of Nichol's study did not yield any evidence of cross resistance between the two drugs thereby differring from the results obtained in falciparum malaria in this study. The main difference between this study and that of Nichol is that in his study the amethopterin sensitive subline was derived from a resistant parent line. It was the opposite in our study. This might suggest that pyrimethamine resistance has a wider range of cross resistance than amethopterin.

Although amethopterin has never been used in malarial chemotherapy, it is not unexpected to find cross resistance between pyrimethamine and amethopterin since the latter compound also inhibits the transfer of hydrogen from NADPH to dihydrofolate (Osborn and Heunnekens, 1958). A remarkable increase in the amount of dihydrofolate reductase has been observed in resistant leukemia cell (Hakala *et al.*, 1961; Hakala and Ishihara, 1962). These observations accorded with the mechanism of resistance to pyrimethamine in *P. falciparum* (Kan and Siddiqui, 1979).

Although the mechanism whereby resistance to sulfonamides is acquired by malarial parasites had been investigated only in the case of sulfadoxine resistance (Tan-ariya and Brockelman, 1983) cross resistance to other sulfonamides might occur because of a similarity in their structures and mode of action. Up to present there has been only one report on cross resistance of sulfadiazine and sulfanilamide in *P. gallinaceum* (Thurston, 1953). In this study, the *in vitro* response of falciparum isolates to three sulfonamides, i.e., sulfadoxine, sulfadiazine and sulfisoxasole, indicated no evidence of cross resistance to sulfadoxine and the other sulfonamides. No relative potencies of three sulfonamides with respect to the isolate and to each other was observed, thus it seemed likely that at this stage there is no cross resistance in *P. falciparum* to the drugs of sulfonamide group which could be shown *in vitro*.

SUMMARY

Cross resistance of pyrimethamine and amethopterin, sulfadoxine and the other sulfonamides in *Plasmodium falciparum* culture lines was studied. Our results indicate some evidence of a cross resistance between pyrimethamine to amethopterin a drug sharing the same mode of action but never been

used as an antimalarial before. Studies on sulfonamides revealed that the minimal inhibitory concentration for sulfadoxine was lower than for sulfadiazine and sulfisoxasole, and that a cross resistance between sulfadoxine and the other sulfonamides may not occur.

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