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Abstract. We have studied the reversal of activity against Plasmodium falciparum of WR99210, a triazine antimarial drug, and of the pro-drug PS-15 by folic acid (FA) and folic acid (FNA). Folic acid and FNA inhibit the growth of P. falciparum in vitro at concentrations > 10^{-4.5} and 10^{-3.5} mol/L, respectively. The activity of pyrimethamine against Kenyan strains M24 and K39 is reduced 10–12-fold by 10^{-5} mol/L of FA, and virtually eliminated by 10^{-5} mol/L of FNA. Folates do not antagonise the action of WR99210 against Kenyan strains, and only partially antagonize the action of WR99210 action against the Southeast Asian strains V1/S and W282. Similarly, FA and FNA exerted weak or no antagonism of the action of PS-15. The inability of folates to antagonize the action of WR99210 can be explained in terms of high drug-enzyme affinity, but this does not account for the inability of FA and FNA to antagonize PS-15. These results suggest that action of PS-15 against P. falciparum is primarily due to a non-folate mechanism.

Chemotherapy remains the mainstay in the control and treatment of malaria. However, parasite resistance to the available antimalarial drugs is spreading rapidly. Chloroquine is now almost totally ineffective in most parts of Africa and reports indicate that resistance to pyrimethamine-sulfadoxine, the current first-line treatment for chloroquine-resistant parasites in Africa, will soon become widespread, rendering the drug useless. Therefore, there is an urgent need for new antimalarial drugs. WR99210 and its prodrug PS-15 represent a new class of highly active antifolate drugs that could be used in combination therapy against multidrug-resistant parasites.

WR99210 (4,6-diamino-1,2-dihydro-2,2-dimethyl-1-[(2,4,5-trichlorophenoxy)propoxy]-1,3,5-triazine), is a close structural analog of the antimalarial antifolate drug cycloguanil, which has pronounced activity in vitro against all Plasmodium falciparum strains studied to date. PS-15 (N-(3-(2,4,5-trichlorophenoxy)propoxy)-N’-(1-methylethyl)-imidocarbonimidodiamide hydrochloride), a close analog of proguanil, is a prodrug of WR99210 developed to overcome the low bioavailability and gastrointestinal intolerance that occurs with oral dosages of WR99210 in humans.

Several factors suggest that both WR99210 and PS-15 have antifolate activity directed against parasite dihydrofolate reductase: the close structural similarity between PS-15 and proguanil, and between WR99210 and cycloguanil; the ability of folic acid (FNA) to reverse the gastrointestinal intolerance caused by WR99210; and the fact that both compounds interact synergistically with sulfonamide. Conversely, the high level of activity of WR99210 against all isolates tested, the lack of cross-resistance with other dihydrofolate reductase (DHFR) inhibitors, and the synergistic interaction between PS-15 and atovoquone, a drug that inhibits parasite mitochondrial electron transport, collectively imply that both WR99210 and PS-15 may inhibit P. falciparum by additional mechanisms. It is currently uncertain whether the inhibition of parasite DHFR is a major component of the action of these drugs. Several reports have demonstrated the ability of P. falciparum to use exogenous folate precursors if the de novo synthesis pathway is blocked, and there is also direct evidence for a folate salvage pathway in P. falciparum. Therefore, if the antimalarial activities of WR99210 and PS-15 involve the inhibition of parasite DHFR, addition of folic acid (FA) to the test system would significantly reduce drug action through competitive antagonism while FNA, by supplying the product of the inhibited enzyme, would completely reverse antifolate drug action. We have used these specific characteristics of FA and FNA to probe the mechanisms of action of PS-15 and WR99210 in vitro experiments. We also attempted to assess to what extent the antimalarial activity of these compounds is attributable to the antifolate or to the non-antifolate mechanism.

MATERIALS AND METHODS

Plasmodium falciparum strains were maintained in continuous culture by standard methods using modified RPMI medium 1640 containing 25 mmol/L of both sodium bicarbonate and HEPES buffer, and 10% normal, pooled human serum, but without FA or p-aminobenzoic acid. Four cloned lines of parasites were studied: M24 and K39 from Kenya, and V1/S and W282 from Southeast Asia. The DHFR genotypes and antifolate sensitivities of the strains are shown in Table 1. All strains except M24 are resistant to chloroquine while W282 is also resistant to quinine.

WR99210 and PS-15, supplied by the Jacobus Pharmaceutical Company (Princeton, NJ), were prepared as stock solutions of 2.42 ng/ml and 270 µg/ml, respectively. Folic acid and FNA, obtained from Sigma (Poole, United Kingdom), were prepared as solutions of 10^{-2} M and stored at −20°C. Final dilutions of drugs and folate antagonists were prepared in culture medium with serum (CMS) that contained < 0.001% ethanol. Concentrations of WR99210 and PS-15 that resulted in 50% inhibition of parasite growth (IC50) were determined by a radioisotope incorporation method. Each test was conducted in triplicate. Briefly, 25 µl of CMS was added to all wells of a 96-well, flat-bottomed
concentration of 10
a preliminary test to validate our methodology, FA at a con-
L of both FA and FNA for the antagonism experiments. In

crations to de-

Comparison to de-

PS-15 is more active than proguanil, which has IC50 values

times more potent than PS-15, as judged by the difference

PS-15

tect signi®cant differences were made using Student’s

WR99210

microculture plate (Sterilin®; Stone, United Kingdom). The

first row of wells constituted control cultures. Twenty-five

microliters of drug in CMS were added to the second row

of wells and serial two-fold dilutions of drug over a 64-fold

range were prepared using a manual microdiluter. Wells were

incubated in an atmosphere of 3% CO2, 5% O2, and 92% N2

at 37°C. After 48 hr, each well was pulsed with 0.5 μCi of

3H-hypoxanthine, incubated for an additional 18 hr, and har-

vested onto filter paper strips for the measurement of radio-
isotope incorporation. The IC50 was determined by interpo-

lation of the log dose/response curve. 16 Comparisons to de-

tect signi®cant differences were made using Student’s t-test

RESULTS

In our test system, PS-15 was a comparatively weak in-
hibitor of P. falciparum in vitro. However, with IC50 values

gainst the four test isolates ranging from 206 to 445 ng/ml,

PS-15 is more active than proguanil, which has IC50 values

in excess of 1

m

PS-15

results have con®rmed previous reports 6 of the com-

highly

antagonism of pyrimethamine with 22-fold and 27-fold increases

in IC50 against the respective strains (Table 3). Both FA and

FNA were weaker antagonists of WR99210 than of pyri-
methamine. The inclusion of FA signi®cantly increased the

IC50 of WR99210 against strains M24 and V1/S, but not

against strains W282 or K39. Folinic acid showed moderate

antagonism of WR99210 against the Southeast Asian strains,

but had no effect on drug action against the African parasites

(Table 3). Folic acid had no detectable effect on the action

of PS-15 against the Kenyan strains (M24 and K39), and

had a significant but weak effect in the case of the Asian

parasites (V1/S and W282). Folinic acid showed a similarly

weak antagonism in the PS-15 tests against the African para-

sites, but not against the African parasites (Table 3).

Drug to folate ratios at the minimum folate concentration

required to cause signi®cant antagonism were calculated for

each of the drug/folate combinations. These ratios, referred

to as critical ratios, ranged between 1:1,105 and 1:135,000

for WR99210, but were much higher for PS-15, ranging be-

tween 1:50 and 250:1. Pyrimethamine had critical ratios sim-

ilar to those of PS-15 (Table 4).

DISCUSSION

Our results have con®rmed previous reports 6 of the com-

paratively weak in vitro activity of PS-15, and the highly

increased activity of the triazine metabolite. Against the V1/


table 1

Dihydrofolate reductase (DHFR) mutation in test strains of Plas-

modium falciparum and antifolate sensitivity 1 8

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mutations in DHFR</th>
<th>Antifolate sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>51</td>
<td>59</td>
</tr>
<tr>
<td>M24‡</td>
<td>Asn Cys Ser Ile</td>
<td>S S S</td>
</tr>
<tr>
<td>K39</td>
<td>Ile Cys Asn Ile</td>
<td>R R R</td>
</tr>
<tr>
<td>W282</td>
<td>Ile Arg Asn Ile</td>
<td>R I R</td>
</tr>
<tr>
<td>V1/S</td>
<td>Ile Arg Asn Leu</td>
<td>R R R</td>
</tr>
</tbody>
</table>

* Values are the 50% inhibitory concentration (IC50) (± SD) in ng/ml.

FIGURE 1. Effect of folic acid (FA) (—■—) and folinic acid (FNA) (—□—) on the growth of Plasmodium falciparum (K39 iso-
late). Growth is shown as a percentage of growth in control culture without folate. Bars show the standard deviation.

TABLE 2

Efficacy of PS-15 and WR99210 against laboratory strains of Plasmodium falciparum*
S strain, antagonism of WR99210 action is apparent at FNA concentrations as low as $10^{-6}$ mol/L, increasing to a maximal effect at $10^{-4.5}$ mol/L, and then apparently decreasing at higher FNA concentrations. It seems likely that this activity profile is biphasic, and that the residual curve represents an interaction of two opposing effects. The first of these is true antagonism of antifolate action, which in the absence of any modifying influence, would increase exponentially with increasing FNA concentration to the point of extinction of drug action. The second effect is FNA toxicity. Figure 1 shows that both FA and FNA are toxic to the growing parasite at concentrations exceeding $10^{-6}$ and $10^{-4.5}$ mol/L, respectively. Since maximum antagonism by FNA of WR99210 action against strains V1/S and W282 was found at a concentration of $10^{-4.5}$ mol/L, the concentration at which FNA toxicity becomes significant, it is likely that the antagonism profiles that we have obtained (Figure 2) represent the combined effect of these two processes on the parasite. Because of the biphasic nature of the antagonism profile, it is impossible to measure accurately the change in IC$_{50}$ associated with complete drug antagonism. However, the 27-fold increase in pyrimethamine IC$_{50}$ produced by $10^{-5}$ mol/L FNA is a measure of complete reversal of antifolate action in this system.

The weak antagonism of WR99210 by FA and FNA may suggest activity directed at an alternative site outside the folate metabolic pathway, although it also could arise from the strength of drug-DHFR enzyme binding or both. McCormick and others reported the failure by FA and FNA to reverse the activity of pyrimethamine, a pure DHFR inhibitor, against P. knowlesi. They suggested that this may reflect poor use of intact folate by Plasmodia. However, the ability of Plasmodia to use intact exogenous folates has since been confirmed, and the observation by McCormick and others more likely reflects a failure to achieve the appropriate folate to drug ratio necessary for significant antagonism to be observed. Yeo and others reversed the activity of WR99210 in the K1 strain of P. falciparum with FNA only at a drug to folate molar ratio > 1:400. In our system, significant FNA antagonism of WR99210 was observed at a drug to folate ratio < 1:1,105 and 1:1,1051 for strains V1/S and W282, respectively. The fact that antagonism was proportional to FNA concentration suggests that at a sufficiently high FNA concentration complete elimination of WR992190 activity can be achieved, but observation of this is obscured by FNA toxicity. Thus, WR99210 may simply be a particularly potent DHFR inhibitor without necessarily bearing a second mechanism.

It is important to note that although both strains W282 and V1/S are equally sensitive to WR99210 in vitro, the critical drug to folate ratio is 10 times higher for strain W282. It is possible that the critical ratios for the Kenyan strains are achieved at folate concentrations that are so high that observation of folate/drug antagonism is obscured by the toxic effects of the folate. On the other hand, critical drug:folate ratios for PS-15 are > 2:1 for FNA and 1:50 for FA. The critical ratios for pyrimethamine are also equally high, reflecting a relatively weaker DHFR binding compared with WR99210. This suggests that folates antagonizes the antifolate function of PS-15 to the same extent as they do to pyrimethamine, but whereas antagonism of pyrimethamine is proportional to the folate concentration, antagonism of PS-15 is not (Figure 3). Thus, it may not be possible to reverse PS-15 activity even at high folate concentrations, suggesting that PS-15 may act against malaria parasites primarily through a non-antifolate mechanism.

Our observation that folates antagonize WR99210 but not PS-15 agrees with the report by Yeo and others and sup-

<table>
<thead>
<tr>
<th>Strain</th>
<th>M24</th>
<th>K39</th>
<th>V1/S</th>
<th>W282</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyrimethamine†</td>
<td>FA</td>
<td>12</td>
<td>10</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>FNA</td>
<td>27.0</td>
<td>22.0</td>
<td>ND</td>
</tr>
<tr>
<td>WR99210</td>
<td>FA</td>
<td>2.05 ± 1.00‡</td>
<td>0.96 ± 0.02</td>
<td>2.73 ± 1.51‡</td>
</tr>
<tr>
<td></td>
<td>FNA</td>
<td>1.41 ± 0.29</td>
<td>1.18 ± 0.01</td>
<td>8.77 ± 1.14‡</td>
</tr>
<tr>
<td>PS-15</td>
<td>FA</td>
<td>1.71 ± 0.84</td>
<td>1.10 ± 0.14</td>
<td>4.12 ± 0.27‡</td>
</tr>
<tr>
<td></td>
<td>FNA</td>
<td>2.09 ± 0.64</td>
<td>1.04 ± 0.09</td>
<td>2.59 ± 1.01‡</td>
</tr>
</tbody>
</table>

* FA = folic acid, ND = not done; FNA = folic acid. Antagonism = 50% inhibition concentration (IC$_{50}$) in media with folate/IC$_{50}$ in media without folate. Values are mean ± SD. † Done in duplicate; the rest were done in triplicate.

**Table 4**

Critical drug:folate ratios (defined in the Results) for various drug/folate combinations

<table>
<thead>
<tr>
<th>Drug</th>
<th>Folate</th>
<th>V1/S</th>
<th>W282</th>
<th>M24</th>
<th>K39</th>
</tr>
</thead>
<tbody>
<tr>
<td>WR99210</td>
<td>FNA</td>
<td>1:1,105</td>
<td>1:11,051</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>FA</td>
<td>1:110,512</td>
<td>NS</td>
<td>1:135,000</td>
<td>NS</td>
</tr>
<tr>
<td>PS-15</td>
<td>FNA</td>
<td>2:1</td>
<td>250:1</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>FA</td>
<td>1:14</td>
<td>1:50</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Pyrimethamine</td>
<td>FNA</td>
<td>ND</td>
<td>ND</td>
<td>1:25</td>
<td>1:2</td>
</tr>
<tr>
<td></td>
<td>FA</td>
<td>ND</td>
<td>ND</td>
<td>2:5</td>
<td>1:1</td>
</tr>
</tbody>
</table>

* FNA = folic acid, NS = no significant (P > 0.05) antagonism of drug activity observed; FA = folic acid; ND = not done.
ports the observation by Fidock and Wellems\textsuperscript{20} that transformation with methotrexate-resistant human DHFR renders malaria parasites insensitive to WR99210 and cycloguanil antifolates without affecting the intrinsic activity of proguanil. Taken together, these observations indicate that the triazines are primarily antifolates while their biguanide prodrugs act through a non-antifolate mechanism. Although the exclusive potentiation by atovoquone of PS-15 and proguanil but not of their metabolites has been reported,\textsuperscript{21} Canfield and others reported the potentiation of cycloguanil by atovoquone against one of their test strains.\textsuperscript{9} Thus, the evidence for the exclusive potentiation by atovoquone of the biguanide prodrugs but not of their triazine metabolites is equivocal. The nature of this secondary activity that characterizes biguanides remains uncertain, although \textit{in vitro} synergy with atovoquone strongly suggests a common site of action.

In view of the rapid emergence and spread of parasite resistance to the currently available antimalarial drugs, there is an urgent need for alternative drugs that have high activity against malaria parasites exhibiting a broad range of drug sensitivity patterns. The use of combination drugs provide alternatives with high activity as a result of synergy between the constituents. PS-15 provides a means of exploiting the high antimalarial activity of WR99210. The possession of an intrinsic mode of action against malaria parasites that is different from that of its metabolite makes PS-15 a candidate for a novel multiple combination antimalarial therapy. A combination of PS-15, atovoquone, and a dihydropteroate synthetase inhibitor such as dapsone can be formulated. Although PS-15 synergizes atovoquone, its metabolite WR99210 is synergistic with dapsone. Besides having high efficacy against all malaria parasites, such a combination would be expected to limit development of resistance to the constituent drugs.

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