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Effects of Dual Combinations of Antifolates with Atovaquone or Dapsone on Nucleotide Levels in *Plasmodium falciparum*

Anthony E. T. Yeo,† Kristen K. Seymour,**
Karl H. Rieckmann† and Richard I. Christopherson‡*

*Department of Biochemistry, University of Sydney, Sydney, NSW 2006, Australia; and the †Army Malaria Research Unit, University of Sydney, Liverpool Military Area, NSW 2174, Australia

ABSTRACT. The triazine antifolates, cycloguanil and 4,6-diamino-1,2-dihydro-2,2-dimethyl-1-[(2,4,5-trichlorophenoxy)propyloxy]-1,3,5-triazine hydrobromide (WR99210), and their parent biguanide compounds, proguanil and *N*-[3-(2,4,5-trichlorophenoxy)propyloxy]-*n*-(1-methylethyl)-imidodicarbonimidic-diamine hydrochloride (PS-15), were tested in combination with a series of antimalarial drugs for synergism against *Plasmodium falciparum* growing in erythrocytic culture. Four synergistic combinations were found: cycloguanil–dapsone, WR99210–dapsone, proguanil–atovaquone, and PS-15–atovaquone. Cycloguanil–dapsone or WR99210–dapsone had a profound suppressive effect on the concentration of dTTP in parasites while that of dATP increased. Depletion of dTTP is consistent with cycloguanil or WR99210 inhibiting dihydrofolate reductase and dapsone inhibiting dihydropteroate synthase. For the combinations proguanil–atovaquone and PS-15–atovaquone, the levels of nucleoside triphosphates (NTPs) and dNTPs were generally suppressed, suggesting that inhibition is not through nucleotide pathways but probably through another metabolic mechanism(s). Combinations of two synergistic pairs of antimalarial drugs, (proguanil–atovaquone)–(cycloguanil–dapsone) and (PS-15–atovaquone)–(WR99210–dapsone), were tested, and it was found that NTPs and dNTPs decreased much more than for a single synergistic combination. Dual synergistic combinations could play an important role in the therapy of multidrug-resistant malaria, just as combination chemotherapy is used to treat cancer. BIOCHEM PHARMACOL 53;7:943–950, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. antifolates; atovaquone; dapsone; malaria; synergism

The biguanide antimalarial drug proguanil is activated by the hepatic mixed-function oxidase system to produce cycloguanil [1]. In *Plasmodium berghei*, cycloguanil inhibits dihydrofolate reductase [2, 3] and is synergistic with sulfonamides, PABA§ analogues that inhibit dihydropteroate synthase (e.g. dapsone [4]). Cycloguanil has a higher affinity for the malarial dihydrofolate reductase than for the mouse enzyme by several hundred-fold [2, 3]. Inhibition of this enzyme would result in decreased levels of one-carbon derivatives of tetrahydrofolate, which would lead ultimately to parasite death.

PS-15, also known as WR250417, was synthesized as a prodrug of WR99210 by analogy with proguanil being converted to the active antifolate cycloguanil [5]. Much attention has been focused on the triazine WR99210, a putative dihydrofolate reductase inhibitor [6], rather than the prodrug PS-15 [5]. WR99210 is a potent antimalarial drug *in vitro*, effective against both chloroquine-resistant and -sensitive *Plasmodium falciparum* [6]. Initial clinical trials with this compound resulted in unacceptable gastrointestinal side-effects as well as poor bioavailability, and further development was abandoned [7]. Formulations and routes of administration were evaluated to enhance absorption and minimize toxic effects. WR99210 is a potentially important antifolate as cross-resistance with other antifolates is not found [8]. It is hoped that PS-15, as a pro-drug, will overcome the patient toxicity of WR99210, but maintain the potent antimalarial activity. The pathway for biosynthesis of folates is shown below in Scheme 1 [9]:

‡ Corresponding author: Richard I. Christopherson, Ph.D., Department of Biochemistry, University of Sydney, Sydney, NSW 2006, Australia. Tel. 61-2-351-6031; FAX 61-2-351-4726; E-mail: r.christopherson@biochem.usyd.edu.au

§ *Abbreviations:* amol/pe, attomoles per parasitized erythrocyte; DHO, dihydroorotate; dNTP, deoxynucleoside triphosphate; FH_2, dihydrofolate; FH_4, tetrahydrofolate; FIC, fractional inhibitory concentration; IC_{50}, the concentration of drug required to suppress 50% of parasite growth compared with controls; LPLF, low *para*-aminobenzoic acid, low folate; PABA, *para*-aminobenzoic acid; MIC, minimum inhibitory concentration; NTP, nucleoside triphosphate; Oro, orotate; pe, parasitized erythrocyte; PS-15 or WR250417, *N*-[3-(2,4,5-trichlorophenoxy)propyloxy]-*n*-(1-methylethyl)-imidodicarbonimidic-diamine hydrochloride; and WR99210, 4,6-diamino-1,2-dihydro-2,2-dimethyl-1-[(2,4,5-trichlorophenoxy)propyloxy]-1,3,5-triazine hydrobromide.

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$$\text{GTP} \xrightarrow{1} \text{H}_2\text{neopterinTP} \xrightarrow{2} \text{H}_2\text{neopterin} \xrightarrow{3}$$
$$\text{H}_2\text{pterinCH}_2\text{OH} \xrightarrow{4} \text{H}_2\text{pterinCH}_2\text{OPP} \xrightarrow{5}$$
$$\text{H}_2\text{pteroate} \xrightarrow{6} \text{FH}_2 \xrightarrow{7} \text{FH}_4$$

Scheme 1.

Until recently, attention has focused on the active antifolate cycloguanil, but with the discovery of potentiation between proguanil and atovaquone observed *in vitro* and in clinical trials [10, 11], proguanil has gained additional importance. Atovaquone, a 2-hydroxy-1,4-naphthoquinone with potent antimalarial activity, acts at the cytochrome bc_1 complex (Complex III) in *P. falciparum* mitochondria, forming a covalent bond with a protein of molecular weight 11,500 [12]. In malarial parasites, electron transport is coupled to dihydroorotate dehydrogenase of *de novo* pyrimidine biosynthesis [13]. Blockade of the *de novo* pyrimidine pathway would lead to deficiencies of pyrimidine nucleotides and impaired DNA synthesis. Parasites are unable to salvage preformed pyrimidines, and blockade of the *de novo* pathway would lead to cell death [14].

With the development of multidrug-resistant *P. falciparum*, combinations of antimalarial drugs are required to kill parasites in an infected individual. This paper focuses on dual combinations of some antimalarial compounds to determine possible synergy. The compounds tested were proguanil, PS-15, cycloguanil, WR99210, atovaquone, dapsone, pyrimethamine, azithromycin, and ciprofloxacin. Pyrimethamine is also an inhibitor of dihydrofolate reductase, azithromycin is an antibiotic with significant antimalarial activity [15], and ciprofloxacin may act as an inhibitor of electron transport or an uncoupler of oxidative phosphorylation in mammalian cells [16] and may be clinically undervalued as an antimalarial drug [17].

The growth and division of *P. falciparum* is dependent upon an adequate supply of NTPs and dNTPs. Determination of the effects on NTP and dNTP levels in *P. falciparum* induced by a drug may provide information on its mechanism of action. Such changes have been interpreted in terms of inhibition of the pathways of nucleotide biosynthesis in *P. falciparum* growing in erythrocytic culture.

MATERIALS AND METHODS

Materials

Atovaquone, cycloguanil, dapsone, proguanil, PS-15, and pyrimethamine were obtained from Jacobus Pharmaceutical Inc. (Princeton, NJ, U.S.A.). Atovaquone was obtained from Wellcome (Beckenham, U.K.). Proguanil and cycloguanil were from ICI (Macclesfield, U.K., and Sydney, Australia), and azithromycin and ciprofloxacin were from Pfizer (Sydney, Australia) and Bayer (Sydney, Australia), respectively. LPLF RPMI was obtained from Gibco (Grand Island, NY, U.S.A.). A gas mixture of 90% N_2, 5% CO_2, and 5% O_2 was obtained from BOC Gases (Sydney, Australia). Flat-bottom 96-well microtitre plates were obtained from Flow Laboratories (McLean, VA, U.S.A.), and flasks were obtained from Corning (Corning, NY, U.S.A.). Erythrocytes were obtained from healthy donors screened negative for HIV and for hepatitis B and C; human serum also screened for the same viral pathogens was obtained from the Red Cross Blood Bank.

Drug Concentrations

Drug dilutions in ethanol were prepared in 96-well microtitre plates at the following final concentrations upon addition of 100 μL of an erythrocyte suspension: atovaquone: 0.546 to 0.00000546 μM; cycloguanil: 5.09 to 0.00397 μM; dapsone: 80.5 to 0.0805 μM; proguanil: 172 to 1.38 μM; PS-15: 22.2 to 0.0443 μM; pyrimethamine: 6.43 to 0.0000401 μM; WR99210: 0.00232 to 0.0000232 μM; azithromycin: 26.7 to 0.0267 μM; and ciprofloxacin: 211 to 0.211 μM. The pre-dosed plates were then dried overnight at 37° in an incubator. Drugs were dissolved in DMSO on the day of the experiment for addition to erythrocytic cultures.

Combinations of Drugs

To analyse the combined effect of two drugs, the concept of fractional inhibitory concentration (FIC) was used [18]. To determine the FIC, the minimum inhibitory concentration (MIC) of a drug was first determined. The FIC was then the actual MIC of one drug in the presence of a second drug, expressed as a fraction of its MIC when used alone. The FIC for each drug combination could then be determined where values of $A_e/A_c + B_e/B_c < 0.25$ indicate synergy [19] A_e and B_e are the concentrations of each drug used in combination and A_c and B_c are the MICs of each drug alone. For example, the FIC of cycloguanil was calculated by dividing the MIC of cycloguanil combined with dapsone by the MIC of cycloguanil alone. Synergism is demonstrated when the line joining the points of an isobologram falls below a line connecting the MIC of each drug alone, giving a concave curve.

Parasites

The multidrug-resistant K1 strain of *P. falciparum* [20] was maintained in low PABA low folate, LPLF-RPMI medium (0.0005 mg/L PABA and 0.01 mg/L folate), 25 mM K·HEPES (pH 7.2), 32 mM $NaHCO_3$, gentamicin (40 μg/mL) in 10% (v/v) human serum [21]. Cultures were maintained at 37.5° in a gas mixture of 5% CO_2, 5% O_2, and 90% N_2 and were synchronized with 5% (w/v) sorbitol [22]. To determine an MIC or an FIC, 100 μL of a culture of 4% haematocrit, 0.5% parasitemia in 50% (v/v) serum was added to each well. The MIC was determined by microscopic examination to the drug concentration where at least 95% of the parasites failed to reinvade [23].

Metabolic Effects of Drugs

Synchronized malarial cultures [4% haematocrit, 5% parasitemia, 10% (v/v) human serum] were used to determine

the effects of drugs on levels of nucleotides. Drug was added to the cultures approximately 24 hr into the 48-hr asexual life cycle where most parasites are in the trophozoite-early schizont stage, and the *de novo* pyrimidine pathway is most active [24]. Parasites were incubated with drug(s) for 6 hr prior to harvesting. To investigate the effects of a synergistic combination such as cycloguanil–dapsone, it was necessary to run the four cultures concurrently: control, dapsone, cycloguanil, and cycloguanil–dapsone.

Preparation of Malarial Extracts

Metabolites were extracted from parasites as described by Seymour *et al.* [24]. All procedures were conducted at 0°; parasitized erythrocytes (50 mL) were harvested by centrifugation (225 *g*, 10 min), and erythrocytes were lysed by vortexing for 1 min in 0.15% (w/v) saponin in Hanks' balanced salt solution (10 mL, pH 7.4) and left on ice for 5 min. The suspension was then diluted to 50 mL with Hanks' balanced salt solution, and freed parasites were collected by centrifugation (1600 *g*, 15 min). The parasites were resuspended twice more, and the final weight of the pellet was used to calculate the volume. Parasites were then frozen in liquid nitrogen, an equal volume of 0.8 M $HClO_4$ was added, and the suspension was thawed briefly at 37°. The suspension was frozen and thawed twice more and then left on ice for 15 min. After centrifugation (1600 *g*, 15 min), the acidic supernatant was neutralized by vortexing for 1 min with an equal volume of 0.5 M trioctylamine in 1,1,2-trichlorotrifluoroethane [25]. The phases were separated again by centrifugation (9000 *g*, 5 min), and the upper aqueous phase was retained for analysis.

High Pressure Liquid Chromatography

Acid-soluble metabolites were separated by gradient anion-exchange HPLC on a Partisil 10-SAX column (0.42 × 22 cm; Whatman Inc., Clifton, NJ, U.S.A.). The mobile phase used was a concave gradient (curve 7, Waters Automated Gradient Controller) from buffer A (7.0 mM KH_2PO_4, pH 3.0) to buffer B (250 mM KH_2PO_4, 500 mM KCl, pH 3.8). Metabolites were quantified using a UV 2000 ultraviolet detector (Spectra-Physics Analytical, San Jose, CA, U.S.A.) set at 260 nm [24]. Metabolite concentrations have been expressed as amol/parasitized erythrocyte (amol/pe) and as a fraction of control parasites.

RESULTS

The levels of NTPs and dNTPs in an untreated erythrocytic culture of *P. falciparum* approximately 24 hr into the asexual life cycle are shown in Table 1. Because the volume of a parasite is not known, nucleotide levels have been expressed as attomoles per parasitized erythrocyte. The standard errors for these levels were small, and it is concluded that this procedure for quantifying NTPs and dNTPs is reliable with values similar to those obtained by Seymour *et al.* [24]. Table 2 shows the MICs of the various antimalarial drugs.

TABLE 1. Levels of NTPs and dNTPs in *P. falciparum* growing in erythrocytic culture

Nucleotide	(amol/pe)	Maximum value	Minimum value
GTP	5.49 ± 0.90	13.8	0.715
ATP	20.2 ± 3.0	51.0	5.18
UTP	5.65 ± 0.67	10.7	1.10
CTP	2.24 ± 0.38	6.22	0.311
dATP	0.303 ± 0.057	0.753	0.057
dTTP	0.377 ± 0.053	0.844	0.096
dCTP	0.543 ± 0.093	1.30	0.051

Parasites (50 mL, 4% haematocrit, 5% parasitemia) were harvested 24 hr into the asexual life cycle and extracted, and nucleotides were quantified by HPLC as described in Materials and Methods. Data (amol/parasitized erythrocyte) are presented as the means ± SEM for 14 different cultures. dGTP, with the longest retention time, could not be detected reliably due to the low concentrations and broad peak.

The following combinations were synergistic: cycloguanil-dapsone and WR99210–dapsone. (Fig. 1). The remaining drugs were not synergistic with cycloguanil: atovaquone, proguanil, PS-15, pyrimethamine, WR99210, azithromycin, and ciprofloxacin. The following drugs were also not synergistic with WR99210: atovaquone, cycloguanil, PS-15, pyrimethamine, azithromycin, and ciprofloxacin.

HPLC profiles for analysis of dNTPs were then obtained, and the various metabolites were measured: peak areas were determined by integration with Nelson software (3000 Series Chromatography Data System; Nelson Analytical Inc., Cupertino, CA, U.S.A.), and data have been presented in tabular form. A paired *t*-test was performed on all metabolite levels comparing single drug with dual drug combinations. These data were statistically significant with a probability of $P \leq 0.05$.

Levels of nucleotides and their relative proportions compared with a control are listed for cycloguanil, dapsone, and cycloguanil–dapsone (Table 3). Cycloguanil (2.5 μM, 6 hr) induced in the parasite a decrease in the level of dTTP and a major accumulation of dATP (Table 3). Dapsone (250

TABLE 2. Minimum inhibitory concentrations of the antimalarial drugs

Drugs	MIC (μM)	N
Atovaquone	0.0401 ± 0.0126	12
Cycloguanil	0.0802 ± 0.0183	7
Dapsone	73.8 ± 6.7	6
Proguanil	29.9 ± 5.1	8
PS-15	4.21 ± 1.04	26
Pyrimethamine	18.7 ± 4.9	7
WR99210	0.000696 ± 0.000209	10
Azithromycin	8.28 ± 3.20	8
Ciprofloxacin	82.1 ± 9.4	9

MIC values were determined by microscopy as described in Materials and Methods. Data are presented as the means ± SEM for the indicated number of experiments (N).

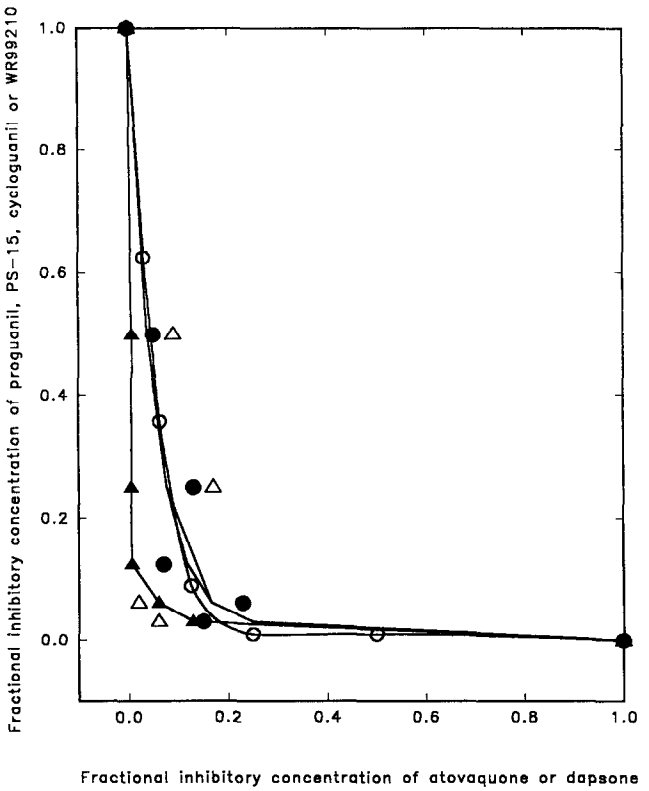

FIG. 1. Isobolograms for synergistic combinations of antimalarial drugs. The FIC of proguanil, PS-15, cycloguanil, or WR99210 (ordinate) were plotted with the FIC of atovaquone or dapsone (abscissa), respectively. Key: (○) proguanil–atovaquone; (△) PS-15–atovaquone; (●) cycloguanil–dapsone; and (▲) WR99210–dapsone.

μM, 6 hr) induced decreases in some dNTPs and NTPs with dTTP decreased to the greatest extent. The combination of cycloguanil–dapsone caused an increase in UTP and CTP with a marked depression of dTTP attributable to concurrent inhibition of dihydropteroate synthase and dihydrofolate reductase. UTP and CTP may accumulate due to the blockade at thymidylate synthase (dUMP → dTMP). The result for UTP was statistically significant ($P < 0.05$) for the dual combination compared with dapsone. The selective effect of cycloguanil–dapsone on dTTP may be attributed to a deficiency of $N^{5,10}$-CH_2-FH_4 required for thymidylate synthase. This decrease in dTTP was statistically significant ($P < 0.05$) compared with the depleted levels determined for cycloguanil or dapsone alone. WR99210 (1 μM, 6 hr), like cycloguanil, induced an accumulation of UTP and CTP and a severe depletion of dTTP (Table 4). The combination of WR99210–dapsone induced further depression of dTTP, and UTP, CTP, and dATP again accumulated. The decrease in dTTP induced by WR99210–dapsone was statistically significant ($P < 0.05$) compared with the level determined for dapsone alone.

The following combinations were synergistic: proguanil–atovaquone and PS-15-atovaquone (Fig. 1). The remaining drugs were not synergistic with proguanil: cycloguanil, dapsone, pyrimethamine, azithromycin, and ciprofloxacin. The following drugs were also not synergistic with PS-15: cycloguanil, dapsone, pyrimethamine, WR99210, azithromycin, and ciprofloxacin. Table 5 summarizes changes induced in the levels of NTPs and dNTPs compared with controls for atovaquone (2.5 μM, 6 hr), proguanil (100 μM, 6 hr), and proguanil–atovaquone (100 and 2.5 μM, respectively, 6 hr). Atovaquone induced decreases in UTP and CTP consistent with inhibition of dihydroorotate dehydrogenase (DHO → Oro) of the *de novo* pyrimidine pathway, while dTTP was depressed and dCTP remained unchanged. Proguanil suppressed most dNTPs and NTPs, indicating a nonspecific effect. The combination of proguanil–atovaquone induced an effect similar to proguanil alone, with decreases in the levels of both dNTPs and NTPs. Table 6 summarizes changes induced in the levels of NTPs and dNTPs, compared with controls, for atovaquone (2.5 μM, 6 hr), PS-15 (10 μM, 6 hr), and PS-15–atovaquone (10 and 2.5 μM, 6 hr). Differences between the dual combination and atovaquone alone were statistically significant ($P < 0.05$) for GTP, ATP, and UTP. PS-15 induced effects similar to proguanil: all dNTPs and NTPs were suppressed. The combination of PS-15–atovaquone induced decreases in all dNTPs and NTPs, similar to PS-15 or proguanil alone. Differences between the dual combination and atovaquone alone were statistically significant ($P < 0.05$) for GTP, ATP, CTP, and dATP. The quadruple combinations, (Proguanil–atovaquone)–(cycloguanil-dapsone) and (PS-15–atovaquone)–(WR99210–dapsone) generally caused greater depression of the levels of NTPs and dNTPs (Table 7) compared with dual drug combinations. dATP and dTTP concentrations fell to 0.18 and 0.13 of controls for proguanil combinations, while for PS-15 combinations they were 0.19 and 0, respectively.

DISCUSSION

Of the various drug combinations examined with the triazines, cycloguanil–dapsone and WR99210–dapsone were synergistic, consistent with the findings of Watkins *et al.* [26] and Winstanley *et al.* [27], who measured the IC_{50} values of chlorcycloguanil–dapsone and WR99210–dapsone, respectively. Cycloguanil inhibits dihydrofolate reductase (reaction 7, Scheme 1, [2, 3]), whereas dapsone inhibits dihydropteroate synthase (reaction 5, Scheme 1, [4]). Both drugs induced a marked depression of dTTP due to decreased availability of $N^{5,10}$-CH_2-FH_4 as a substrate for thymidylate synthase. CTP and UTP may accumulate due to the inhibition of thymidylate synthase (reaction 7, dUMP → dTMP, Scheme 2) as dUMP and more proximal intermediates accumulate.

$$\begin{array}{l} \text{UMP} \xrightarrow{1} \text{UDP} \xrightarrow{2} \text{UTP} \xrightarrow{3} \text{CTP} \\ \qquad\quad \overset{4}{\downarrow} \\ \qquad\quad \text{dUDP} \xrightarrow{5} \text{dUTP} \xrightarrow{6} \text{dUMP} \xrightarrow{7} \text{dTMP} \xrightarrow{8} \text{dTDP} \\ \qquad\quad \xrightarrow{9} \text{dTTP} \end{array}$$

Scheme 2.

TABLE 3. Effects of cycloguanil (2.5 μM, 6 hr), dapsone (250 μM, 6 hr) and cycloguanil-dapsone (2.5 and 250 μM, respectively, 6 hr) upon levels of NTPs and dNTPs in *P. falciparum*

Nucleotide	Control (amol/pe)	Cycloguanil (fraction of control)		Dapsone (fraction of control)		Cycloguanil-dapsone (fraction of control)	
		Mean	Range	Mean	Range	Mean	Range
GTP	3.48	1.39	0.80	0.903	0.64	1.34	0.18
ATP	15.1	1.45	1.07	1.27	1.45	1.23	0.37
UTP	3.25	2.41	2.39	1.13	1.11	2.06	1.49
CTP	1.19	2.26	2.90	0.966	1.193	1.97	1.61
dATP	0.0634	3.39	2.68	0.946	0.315	1.59	1.25
dTTP	0.226	0.267	0.181	0.766	0.764	0.0574	0.0265
dCTP	0.407	0.859	0.494	1.09	1.26	0.996	1.40

Drugs were dissolved in DMSO, and 40 μL of DMSO–drug mixture was added to a 50-mL culture (4% haematocrit, 5% parasitemia). Data were obtained by procedures outlined in Materials and Methods and are expressed as the means and range for two experiments.

The other antimalarial drugs do not show any synergistic activity with the triazine antifolates because they do not act on the folate pathway or thymidylate synthase. For example, atovaquone has potent antimalarial activity and acts at cytochrome bc_1 (Complex III) of the electron transport chain [12], resulting in inhibition of dihydroorotate dehydrogenase of the *de novo* pyrimidine pathway [28]. But the combination of a triazine antifolate with atovaquone was not synergistic. Neither proguanil nor PS-15 affected the activity of cycloguanil. Pyrimethamine and cycloguanil inhibit dihydrofolate reductase, thus the absence of potentiation observed between pyrimethamine and cycloguanil for *P. falciparum* [29]. Azithromycin is the prototype of a new class of antimicrobial agents, which also has anti-protozoan activity [30] and is active *in vitro* against *P. falciparum* [31] and *in vivo* against *P. berghei* [32]. A recent clinical trial with this compound has also shown it to be effective against *P. falciparum* malaria [15]. However, azithromycin in combination with cycloguanil was not synergistic. Ciprofloxacin, a 4-quinolone, binds to DNA, resulting in more potent inhibition of bacterial DNA topoisomerases compared with mammalian DNA topoisomerases [33] at concentrations achieved *in vivo*. Forsgren *et al.* [34] demonstrated inhibition of *de novo* pyrimidine biosynthesis by ciprofloxacin, resulting in a compensatory increase in the uptake of pyrimidine precursors through salvage pathways. Lyons and Christopherson [16], however, suggest that ciprofloxacin may act as an inhibitor of electron transport or an uncoupler of oxidative phosphorylation in mammalian cells in a fashion analogous to dichloroallyl lawsone [35]. In short, the mechanism of action of ciprofloxacin is not clear. No synergy between ciprofloxacin and cycloguanil was seen.

PS-15, an antimalarial pro-drug, is converted to WR99210, which is more potent than cycloguanil [6]: a lower concentration of WR99210 (1 μM) compared with cycloguanil (2.5 μM) induced a greater depression of dTTP (Tables 3 and 4). Parasitological experiments show that WR99210 behaves like cycloguanil with potentiation of antimalarial activity occurring only with dapsone. As WR99210 has been proposed as a dihydrofolate reductase inhibitor [5], the potentiation of activity would occur via the mechanism proposed above for cycloguanil–dapsone. Atovaquone induces inhibition of dihydroorotate dehydrogenase of the *de novo* pyrimidine pathway and is only ad-

TABLE 4. Effects of WR99210 (1 μM, 6 hr), dapsone (250 μM, 6 hr) and WR99210–dapsone (1 and 250 μM, respectively, 6 hr) upon levels of NTPs and dNTPs in *P. falciparum*

Nucleotide	Control (amol/pe)	WR99210 (fraction of control)		Dapsone (fraction of control)		WR99210–dapsone (fraction of control)	
		Mean	Range	Mean	Range	Mean	Range
GTP	3.98	1.04	1.41	0.741	1.04	1.15	1.47
ATP	17.4	1.15	1.42	0.889	1.14	1.21	1.27
UTP	3.94	2.39	2.70	1.04	1.22	2.20	2.27
CTP	1.56	2.17	2.99	0.788	1.13	2.11	2.41
dATP	0.164	2.10	1.77	0.854	0.366	1.71	0.73
dTTP	0.288	0.0894	0.168	0.833	0.972	0.0531	0.102
dCTP	0.321	1.20	1.78	0.874	1.31	1.19	2.06

Drugs were dissolved in DMSO, and 40 μL of DMSO–drug mixture was added to a 50-mL culture (4% haematocrit, 5% parasitemia). Data were obtained by the procedures outlined in Materials and Methods and are expressed as the means and range for two experiments.

TABLE 5. Effects of atovaquone (2.5 μM, 6 hr), proguanil (100 μM, 6 hr) and proguanil–atovaquone (100 and 2.5 μM, respectively, 6 hr) upon levels of NTPs and dNTPs in *P. falciparum*

Nucleotide	Control (amol/pe)	Atovaquone (fraction of control)		Proguanil (fraction of control)		Proguanil-atovaquone (fraction of control)	
		Mean	Range	Mean	Range	Mean	Range
GTP	4.85	0.923	0.299	0.487	0.069	0.586	0.136
ATP	19.9	0.999	0.132	0.496	0.068	0.523	0.010
UTP	5.06	0.738	0.007	0.465	0.227	0.335	0.084
CTP	1.50	0.864	0.361	0.491	0.044	0.519	0.163
dATP	0.209	1.86	1.47	0.789	1.096	0.596	1.07
dTTP	0.381	0.542	0.256	0.516	0.038	0.308	0.015
dCTP	0.392	1.00	1.33	1.32	2.10	0.819	0.967

Drugs were dissolved in DMSO, and 40 μL of DMSO–drug mixture was added to a 50-mL culture (4% haematocrit, 5% parasitemia). Data were obtained by the procedures outlined in Materials and Methods and are expressed as the means and range for two experiments.

ditive with WR99210 which inhibits dihydrofolate reductase. Pyrimethamine is not synergistic with WR99210 because they both affect dihydrofolate reductase. The antimalarial properties of cycloguanil–dapsone are the same as those of WR99210–dapsone. Cycloguanil, WR99210, and the combinations of cycloguanil–dapsone and WR99210–dapsone induced similar effects on NTPs and dNTPs (Tables 3 and 4), suggesting similar mechanisms of action.

With the biguanides, proguanil and PS-15, only the combinations of proguanil–atovaquone and PS-15–atovaquone were synergistic. The mechanism of toxicity for proguanil is not known. Canfield *et al.* [10] suggested that atovaquone may also have an antifolate effect, and the suppression of dTTP observed here is consistent with this proposal. Proguanil–atovaquone is the latest antimalarial combination used in clinical trials, and our findings are consistent with those of Canfield *et al.* [10] supporting the effectiveness of this combination. Unfortunately, measurements of NTP and dNTP levels in *P. falciparum* do not provide a rationale for the synergistic effect of proguanil–atovaquone (Table 5). PS-15 had the same non-specific effect as proguanil in decreasing NTPs and dNTPs in the parasites (Table 6), suggesting that the mechanism of action does not involve nucleotide biosynthetic pathways. Proguanil, PS-15, and the combinations of proguanil–atovaquone and PS-15–atovaquone induced similar changes in dNTPs and NTPs, suggesting common aspects in their mechanisms of action. The synergistic combination of proguanil–atovaquone is now undergoing Phase 3 clinical trials [10]; a PS-15–atovaquone combination could follow a similar course.

The quadruple combinations of (proguanil–atovaquone)–(cycloguanil–dapsone) and (PS-15–atovaquone)–(WR99210–dapsone) further suppressed levels of NTPs and dNTPs with major decreases in the levels of dATP and dTTP. The use of such quadruple combinations may prevent or, at least, delay the development of parasite resistance to these drugs, but the use of quadruple combinations against drug-resistant malaria remains to be tested in clinical trials. However, possible drug toxicity to patients may limit the clinical usefulness of this combination chemotherapy, and the biguanides would be converted to triazines. In addition to its use against malaria, such combinations could be useful against other parasites such as

TABLE 6. Effects of atovaquone (2.5 μM, 6 hr), PS-15 (10 μM, 6 hr) and PS-15–atovaquone (10 and 2.5 μM, respectively, 6 hr) upon levels of NTPs and dNTPs in *P. falciparum*

Nucleotide	Control (amol/pe)	Atovaquone (fraction of control)		PS-15 (fraction of control)		PS-15-atovaquone (fraction of control)	
		Mean	Range	Mean	Range	Mean	Range
GTP	5.59	0.875	0.563	0.188	0.049	0.253	0.043
ATP	22.1	0.844	0.724	0.252	0.135	0.346	0.031
UTP	5.92	0.425	0.511	0.220	0.017	0.136	0.088
CTP	2.52	0.451	0.265	0.235	0.192	0.110	0.178
dATP	0.196	1.32	0.02	0.153	0.153	0.375	0.417
dTTP	0.367	0.330	0.296	0.182	0.094	0.102	0.051
dCTP	0.654	0.891	0.055	0.501	0.006	0.669	0.407

Drugs were dissolved in DMSO, and 40 μL of DMSO–drug mixture was added to a 50-ml culture (4% haematocrit, 5% parasitemia). Data were obtained by the procedures outlined in Materials and Methods and are expressed as the means and range for two experiments.

TABLE 7. Effects of (proguanil–atovaquone)–(cycloguanil–dapsone) (100, 2.5, 2.5 and 250 μM, respectively, 6 hr) and (PS-15–atovaquone)–(WR99210–dapsone) (10, 2.5, 1 and 250 μM, respectively, 6 hr) combinations upon levels of NTPs and dNTPs in *P. falciparum*

Nucleotide	Control (amol/pe)	(Proguanil–atovaquone)–(cycloguanil–dapsone) (fraction of control)	Control (amol/pe)	(PS-15–atovaquone)–(WR99210–dapsone) (fraction of control)
GTP	4.63	0.401	7.25	0.122
ATP	21.6	0.392	29.6	0.239
UTP	4.48	0.509	6.78	0.364
CTP	1.86	0.429	2.80	0.251
dATP	0.0644	0.181	0.189	0.189
dTTP	0.334	0.132	0.421	0
dCTP	0.660	0.822	0.594	0.525

Drugs were dissolved in DMSO, and 40 μL of DMSO–drug mixture was added to a 50-mL culture (4% haematocrit, 5% parasitemia). Data (results of one experiment) were obtained by the procedures outlined in Materials and Methods.

Pneumocystis carinii and *Toxoplasmosis gondii* in immunocompromised patients.

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