

Rational Drug Design Approach for Overcoming Drug Resistance: Application to Pyrimethamine Resistance in Malaria

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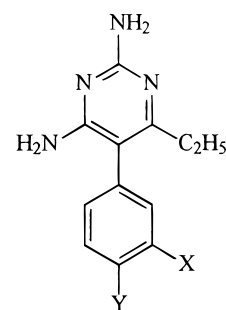
Pyrimethamine acts by selectively inhibiting malarial dihydrofolate reductase–thymidylate synthase (DHFR–TS). Resistance in the most important human parasite, *Plasmodium falciparum*, initially results from an S108N mutation in the DHFR domain, with additional mutation (most commonly C59R or N51I or both) imparting much greater resistance. From a homology model of the 3-D structure of DHFR–TS, rational drug design techniques have been used to design and subsequently synthesize inhibitors able to overcome malarial pyrimethamine resistance. Compared to pyrimethamine (K_i 1.5 nM) with purified recombinant DHFR from *P. falciparum*, the K_i value of the *m*-methoxy analogue of pyrimethamine was 1.07 nM, but against the DHFR bearing the double mutation (C59R + S108N), the K_i values for pyrimethamine and the *m*-methoxy analogue were 71.7 and 14.0 nM, respectively. The *m*-chloro analogue of pyrimethamine was a stronger inhibitor of both wild-type DHFR (with K_i 0.30 nM) and the doubly mutant (C59R + S108N) purified enzyme (with K_i 2.40 nM). Growth of parasite cultures of *P. falciparum* in vitro was also strongly inhibited by these compounds with 50% inhibition of growth occurring at 3.7 μ M for the *m*-methoxy and 0.6 μ M for the *m*-chloro compounds with the K1 parasite line bearing the double mutation (S108N + C59R), compared to 10.2 μ M for pyrimethamine. These inhibitors were also found in preliminary studies to retain antimalarial activity in vivo in *P. berghei*-infected mice.

Introduction

Pyrimethamine (Chart 1) has been employed for many years against malaria, initially on its own but subsequently in combinations such as Maloprim (pyrimethamine–dapson)¹ and Fansidar (pyrimethamine–sulfadoxine), the latter a widely used formulation for the treatment of chloroquine-resistant malaria. Pyronaridine, a new Chinese drug, has been tested against malaria in combination with pyrimethamine.² Pyrimethamine has also been used recently against toxoplasmic encephalitis relapses and *Pneumocystis carinii* pneumonia in HIV-infected patients,^{3,4} and atovaquone has been tested against murine toxoplasmosis in combination with pyrimethamine.⁵ Pyrimethamine also has antibacterial value. Dapsone–pyrimethamine may prevent mycobacterial disease (*Mycobacterium tuberculosis*, *Mycobacterium avium*) in immunosuppressed patients infected with HIV.⁶

With very few safe, effective, and cheap antimalarial drugs, the problem of parasite resistance has enormous economic and social consequences, particularly in Africa, where malaria causes ca. 2 million deaths per year and

Chart 1



	X =	Y =
Pyrimethamine	H	Cl
CC83	OCH ₃	H
SO3	Cl	H

very high levels of morbidity. As a result, ways are being sought to impede the onset and spread of drug resistance. Resistance to pyrimethamine, first reported shortly after its introduction in the 1950s, is now widespread worldwide. To delay (but not overcome) further malarial resistance to inhibitors such as pyrimethamine, combinations such as those above with sulfonamides or sulfones have been in use for some years. Fansidar, for instance, is currently the first-line drug of choice in a

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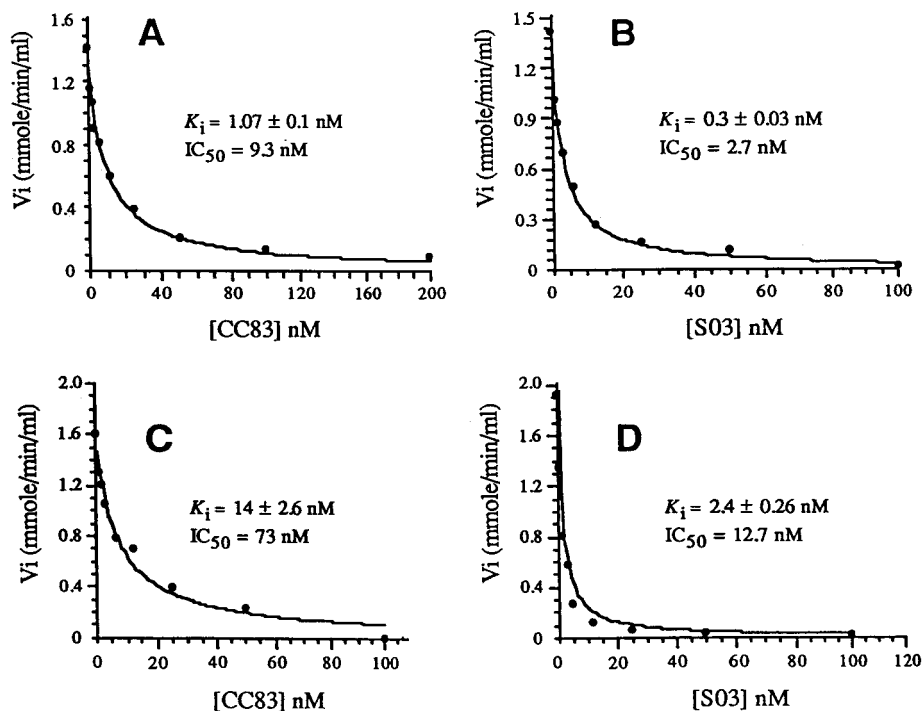


Figure 1. Inhibition of recombinant WT and C59R + S108N mutant DHFRs by the Pyr analogues CC83 and S03. The initial velocities (V_i) were determined in the presence of varying concentrations of inhibitors. The data were fitted using a nonlinear least-squares fit to the equation $IC_{50} = K_i(1 + [S]/K_m)$: (A) inhibition of WT DHFR-TS by CC83, (B) inhibition of WT DHFR-TS by S03, (C) inhibition of mutant DHFR-TS by CC83, and (D) inhibition of mutant DHFR-TS by S03.

number of African countries, but resistance to both components of this combination is an increasing problem.

Pyrimethamine inhibits dihydrofolate reductase-thymidylate synthase (DHFR-TS) in the folate biosynthetic pathway, and resistance to it arises from mutation in the *dhfr-ts* gene. Studies on long-term continuous culture isolates have shown that in the lethal species of the human malaria parasite, *Plasmodium falciparum*, resistance results in the first instance from an S108N mutation in the DHFR domain, but double and triple mutations impart much higher levels of resistance.⁷⁻¹¹ Resistance to pyrimethamine and the related antifolate cycloguanil has recently been studied in a wide range of field samples of *P. falciparum*,¹²⁻¹⁴ as well as in the long-term continuous culture isolates referred to above. The most common resistant strains showed the double mutations S108N + C59R or S108N + N51I or the triple combination of these modifications. Additionally an I164L mutation has been seen in some cases, imparting very high level resistance to both pyrimethamine and cycloguanil. The association of antifolate resistance with mutations in the DHFR domain has been directly demonstrated in vitro via mutagenesis of synthetic *P. falciparum dhfr* genes,^{15,16} as well as by transformation of sensitive parasites with recombinant gene constructs carrying the individual mutations.¹⁷ Further, in multiple mutants there is evidence that these point mutations interact with one another in a cooperative manner.¹⁸ There has been a recent report of the use of computational docking of commercially available compounds to a homology-built molecular model of the DHFR domain of the DHFR-TS of *P. falciparum*.¹⁹ This provided two new families of inhibitors, in which the best K_i achieved was 0.54 μ M against recombinant *P. falciparum* DHFR domain.

One of us recently modeled the three-dimensional structure for DHFR-TS from *Leishmania major*,²⁰ and although this is now superseded by an X-ray diffraction study of this enzyme,²¹ we did not have access to the coordinates at the time of this study. Even using the crude approximation of the *L. major* DHFR-TS model (as a basis for *P. falciparum* DHFR-TS), we were able to design inhibitors with the potential to overcome malarial pyrimethamine resistance. The molecular model indicated for the S108N mutation that, if the active-site structure of *Plasmodium* DHFR-TS was similar to that of *Leishmania*, there would be a steric clash of the protein with the *p*-Cl atom of pyrimethamine. It was proposed, and a molecular graphics picture showed²⁰ that a suitable substituent in the adjacent meta position would avoid this clash and permit an additional interaction with the enzyme. To test this we synthesized two analogues of pyrimethamine, viz., CC83 and S03 (Chart 1), and compared them to the parent compound in three independent assays, using purified recombinant enzymes, in vitro parasite cultures, and in vivo drug challenge.

Results and Discussion

CC83 and S03 were found to be strong inhibitors of DHFRs from *P. falciparum*, plots being shown in Figure 1. The kinetics and inhibition data for DHFR from wild-type (WT) and C59R + S108N double mutant are summarized in Table 1. As reported¹⁸ for purified DHFR-TS (Table 1), C59R-S108N mutations were found to increase K_i for Pyr and Cyc by 48- and 32-fold, respectively, compared to WT. CC83 was found to be 50% more effective as Pyr against WT DHFR-TS, while S03 inhibited WT enzyme 3-fold better than CC83 and 5 and 9 times better than Pyr and Cyc, respectively.

Table 1. Inhibition of Recombinant WT and Mutant (C59R + S108N) *P. falciparum* DHFR-TS by Pyrimethamine (Pyr), Cycloguanil (Cyc), CC83, and S03

parameter	WT enzyme	double-mutant (C59R + S108N)	ratio (mutant/WT)
substrates			
H ₂ Folate, <i>K_m</i> , μM	12.9 ± 4.9	23.7 ± 9.3	1.83
NADPH, <i>K_m</i> , μM ^a	4.7 ± 1.3	14.5 ± 0.8	3.09
inhibitors			
Pyr, <i>K_i</i> , nM ^a	1.5 ± 0.2	71.7 ± 2.9	47.8
Cyc, <i>K_i</i> , nM ^a	2.6 ± 0.3	82.1 ± 3.7	31.5
CC83, <i>K_i</i> , nM ^b	1.07 ± 0.10	14.0 ± 2.6	13.1
S03, <i>K_i</i> , nM ^b	0.30 ± 0.03	2.40 ± 0.26	8.0

^a Data from ref 18. ^b From duplicate experiments.

Table 2. Inhibition of in Vitro *P. falciparum* Parasite Cultures by Pyrimethamine (Pyr), CC83, and S03

	parasite line (IC ₅₀ , μM)	
	HB3 (S108N)	K1(S108N + C59R)
Pyr ^a	1.2	10.2
CC83	0.2	3.7
S03	0.04	0.6

^a IC₅₀ values were defined as the concentration of inhibitor at which parasite growth was reduced to one-half that of mock-treated controls, as measured by parasitemia levels. Average of duplicate determinations.

CC83 and S03 were much stronger inhibitors of the clinically important, double-mutant DHFR. Thus CC83 inhibited resistant enzyme (*K_i* ~14 nM) 5–6-fold better than Pyr or Cyc. S03 was even stronger, being 30–35-fold better than Pyr and Cyc against resistant enzyme. At this purified enzyme level, shifting the substituent of Pyr to give CC83 and S03 did indeed effect the change in inhibition characteristics predicted. The *K_i* for S03 with the doubly mutant DHFR, of the same magnitude as the *K_i* for the therapeutic Pyr and Cyc with WT DHFR, makes S03 a potential drug candidate in resistant cases.

In parasite cultures, IC₅₀ values measured for Pyr, CC83, and S03 against the singly mutated (S108N) HB3 line and doubly mutated (S108N + C59R) K1 line (Table 2) in both cases showed markedly increasing efficacy from Pyr to CC83 to S03. The IC₅₀ for S03 against the moderately resistant HB3 was 40 nM, close to that of Pyr against WT parasites.²² S03 was ~30-fold stronger against HB3 than Pyr. Importantly, S03 was also ~20-fold better than Pyr against the highly resistant, doubly mutated K1 line.

Limited in vivo studies showed CC83 active in vivo against *P. berghei* infection in MF1 mice, which 72 h postinjection (CC83, 5 mg/kg sc twice daily, total 5 doses) showed 3.3 ± 0.3% (standard error of the mean) parasitemia (background Leishman's parasitemia staining, ~1%), compared to vehicle-treated mice with 53.3 ± 6.8% (*P* < 0.001). There were no observable signs of distress or toxicity at this dose in the CC83-treated group over the 3 days (or at 25 mg/kg twice daily for 3 days). Against this *P. berghei* (chloroquine-sensitive) strain, chloroquine diphosphate is active at 2–3 mg/kg. The ID₅₀ (the dose decreasing parasitemia by 50%) for CC83 was estimated as ≤1 mg/kg. Against *P. berghei*-infected mice (5 mg/kg, 5 sc injections of S03 over 3 days) S03 reduced parasitemia from 49.6 ± 9.1% in vehicle-treated malarial mice to 5.1 ± 2.0% (*P* < 0.001).

The strong activity of CC83 and S03 against recombinant DHFR domain of malarial bifunctional DHFR-TS supports the applicability of even the admittedly crude model of the active site used. While modeling predicted that either CC83 or S03 would inhibit S108N mutant DHFR, it did not indicate that S03 would be stronger than CC83 against the double-mutant DHFR (S108N + C59R). This may be related to the cooperativity of interaction of the point-mutation sites in multiple mutants of *P. falciparum* DHFR.¹⁸ Cooperative interactions within the receptor target site clearly must be addressed in general, not just for DHFR, for rational drug design based on proteins as drug targets to be predictive. Other mutations, also known to be involved in resistance,^{7–11} should ideally be included in such analysis.

Rational design methods have only rarely been used to reach testable molecules in parasitology.²³ The present case provides an example of computer-aided drug re-engineering to overcome drug resistance. While current rational drug design methods do not explicitly consider pharmacokinetics or pharmacodynamics, the minimal nature of the physicochemical change relative to pyrimethamine needed for S03 to overcome resistance augurs well for these aspects.

Experimental Section

For synthesis anhydrous reactants and dry solvents were used. Aluminum oxide (active neutral, Brockmann Grade 1) was purchased from BDH Chemicals Ltd. TLC was carried out on alumina and silica plates. Spots were made visible by UV light or exposure to iodine vapor. Melting points, taken on a Gallenkamp apparatus with digital thermometer, are uncorrected. NMR proton spectra were recorded on a JEOL JNM Ex 270 spectrometer operating at 270 MHz. Chemical shifts (δ) are reported in parts per million (ppm) relative to tetramethylsilane or to the 7.25 ppm residual chloroform peak for ¹H NMR spectra. An asterisk indicates a D₂O-exchangeable resonance. Mass spectra were taken by means of a Kratos MS20 instrument coupled to a DS55 data system for precise mass determination. Elemental analyses were performed on an EA 1108 elemental analyzer (Carlo Erba Instruments) in the Department of Chemistry of the University of Manchester.

Synthesis. CC83 and S03 were prepared by literature methods.^{24–26} S03 was isolated as a white powder, which did not need recrystallization: mp 215–220 °C (uncorrected); TLC *R_f* 0.51 in petroleum ether/ethyl acetate (8:2, v/v); NMR (CDCl₃, 270 MHz) δ_H 8.32 (1H, s, ArH), 7.40 (2H, m, ArH), 7.2 (2H, m, ArH), 5.9* (2H, brs, NH₂), 5.6* (2H, brs, NH₂), 2.1 (2H, q, CH₂), 1.0 (3H, t, CH₃); IR (ν, cm⁻¹), 3310 (NH stretch), 1600 (C=N stretch); MS calculated for C₁₂H₁₃ClN₄ 248.7163, found 248.0824. Anal. (C₁₂H₁₃ClN₄) C, H, N.

CC83 was isolated as a white powder which did not need recrystallization: mp 242–244 °C; NMR (DMSO-*d*₆, 270 MHz) δ_H 7.39 (1H, m, Ar-H), 6.97 (1H, m, Ar-H), 6.77 (2H, m, Ar-H), 5.88* (2H, brs, NH₂), 5.46* (2H, brs, NH₂), 3.87 (3H, s, OCH₃), 2.17 (2H, q, CH₂), 1.08 (3H, t, CH₃); IR (ν, cm⁻¹) 3320 (NH stretch); MS calculated for C₁₃H₁₆N₄O 244.2979, found 244.1329. Anal. (C₁₃H₁₆N₄O) C, H, N.

Enzyme Assays. The compounds were tested for the ability to inhibit DHFR activity and parasite growth in culture. The synthetic genes encoding WT and C59R + S108N mutant DHFR domain¹⁸ of bifunctional DHFR-TS were used, and the DHFR activity was determined spectrophotometrically as described.¹⁵ The assay reaction (1 mL) contained 100 μM each of dihydrofolate and NADPH, 50 mM TES, pH 7.0, 75 mM 2-mercaptoethanol, 1 mg/mL bovine serum albumin, varying concentration of inhibitor, and appropriate amount of enzyme. For inhibition studies, CC83 and S03 were initially dissolved in HPLC-grade DMSO and subsequently added to the assay

reaction (final concentrations, 0–1 μ M). The reactions were initiated with purified, recombinant enzymes (~0.6–1 nM). The K_i values were determined by fitting the data to $IC_{50} = K_i(1 + [S]/K_m)$, where K_m is the Michaelis constant for dihydrofolate, $[S]$ is the concentration of dihydrofolate, and IC_{50} is the concentration of inhibitor giving 50% inhibition under the assay conditions described above.

In Vitro Parasite Cultures. In vitro parasite cultures (initial parasitemias, 0.5%) were maintained as described²⁷ but in microtiter plates. Growth, monitored as percentage of parasitemia for a range of inhibitor concentrations on the third and fourth days after adding drug, was compared to mock-treated controls to derive IC_{50} values, with medium and drug replenished on day 2.

In Vivo Studies. *P. berghei* (N/13/1A/4/203), maintained by serial passage in MF1 mice, was injected intravenously (2×10^7 parasitized erythrocytes/animal) into experimental male MF1 mice (initial weight, 18–25 g). In a blind screen, putative antimalarials or vehicle (sterile olive oil and dimethyl sulfoxide, 24:1; dose, 10 mL/kg) were injected subcutaneously into groups of 6 mice. Antimalarials were first injected ~3 h after *P. berghei* inoculation and then twice daily for 2 days. Body weights and colonic temperatures of mice were measured 72 h after inoculation, and parasitemia was assessed from blood smears as the percentage of erythrocytes containing Leishman-positive bodies by light microscopy. The general autonomic and behavioral states of the mice were visually observed throughout the screening period.

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References

- Pividal, J.; Viktinski, V.; Streat, E.; Schapira, A. Efficacy of dapson with pyrimethamine (Maloprim) for malaria prophylaxis in Maputo, Mozambique. *East Afr. Med. J.* **1992**, *69*, 303–305.
- Chen, C.; Zheng, X. Development of the new antimalarial drug pyronaridine: a review. *Biomed. Environ. Sci.* **1992**, *5*, 149–160.
- Ruf, B.; Schurmann, D.; Bergmann, F.; Schuler-Maue, W.; Grunewald, T.; Gottschalk, H. J.; Witt, H.; Pohle, H. D. Efficacy of pyrimethamine/sulfadoxine in the prevention of toxoplasmic encephalitis relapses and pneumocystis carinii pneumonia in HIV-infected patients. *Eur. J. Clin. Microbiol. Infect. Dis.* **1993**, *12*, 325–329.
- Podzamczar, D.; Salazar, A.; Jimenez, J.; Consiglio, E.; Santin, M.; Casanova, A.; Rufi, G.; Gudiol, F. Intermittent trimethoprim-sulfamethoxazole compared with dapson-pyrimethamine for the simultaneous primary prophylaxis of Pneumocystis pneumonia and toxoplasmosis in patients infected with HIV. *Ann. Int. Med.* **1995**, *122*, 755–761.
- Araujo, F. G.; Lin, T.; Remington, J. S. The activity of atovaquone (566C80) in murine toxoplasmosis is markedly augmented when used in combination with pyrimethamine or sulfadiazine. *J. Infect. Dis.* **1993**, *167*, 494–497.
- Opravil, M.; Pechère, M.; Lazzarin, A.; Heald, A.; Rüttimann, S.; Iten, A.; Furrer, H.; Oertle, D.; Praz, G.; Vuitton, D. A.; Hirschel, B.; Lüthy, R. Dapsone/Pyrimethamine may prevent mycobacterial disease in immunosuppressed patients infected with the human immunodeficiency virus. *Clin. Infect. Dis.* **1995**, *20*, 244–249.
- Thaithong, S.; Chan, S. W.; Songsomboon, S.; Wilairat, P.; Seesod, N.; Sueblinwong, T.; Goman, M.; Ridley, R.; Beale, G. Pyrimethamine resistant mutations in *Plasmodium falciparum*. *Mol. Biochem. Parasitol.* **1992**, *52*, 149–157.
- Hyde, J. E. The dihydrofolate reductase-thymidylate synthetase gene in the drug resistance of malaria parasites. *Pharmacol. Ther.* **1990**, *48*, 45–59.
- Sirawaraporn, W.; Sirawaraporn, R.; Cowman, A. F.; Yuthavong, Y.; Santi, D. V. Heterologous expression of active thymidylate synthase-dihydrofolate reductase from *Plasmodium falciparum*. *Biochemistry* **1990**, *29*, 10779–10785.
- Cowman, A. F.; Morry, M. J.; Biggs, B. A.; Cross, G. A.; Foote, S. J. Amino acid changes linked to pyrimethamine resistance in the dihydrofolate reductase-thymidylate synthase gene of *Plasmodium falciparum*. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 9109–9113.
- Peterson, D. S.; Walliker, D.; Wellem, T. E. Evidence that a point mutation in dihydrofolate reductase-thymidylate synthase confers resistance to pyrimethamine in falciparum malaria. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 9114–9118.
- Basco, L. K.; Eldin de Pécoulas, P.; Wilson, C. M.; Le Bras, J.; Mazabraud, A. Point mutations in the dihydrofolate reductase-thymidylate synthase gene and pyrimethamine and cycloguanil resistance in *Plasmodium falciparum*. *Mol. Biochem. Parasitol.* **1995**, *69*, 135–138.
- Wang, P.; Lee, C.; Bayoumi, R.; Djimde, A.; Doumbo, O.; Swedberg, G.; Dao, L. D.; Mshinda, H.; Tanner, M.; Watkins, W. M.; Sims, P.; Hyde, J. E. Resistance to antifolates in *Plasmodium falciparum* monitored by sequence analysis of dihydropteroate synthetase and dihydrofolate reductase alleles in a large number of field samples of diverse origins. *Mol. Biochem. Parasitol.* **1997**, *89*, 161–177.
- Zindrou, S.; Dung, N. P.; Sy, N. D.; Skold, O.; Swedberg, G. *Plasmodium falciparum*: mutation pattern in the dihydrofolate reductase-thymidylate synthase genes of Vietnamese isolates, a novel mutation, and coexistence of two clones in a Thai patient. *Exp. Parasitol.* **1996**, *84*, 56–64.
- Sirawaraporn, W.; Prapunwattana, P.; Sirawaraporn, R.; Yuthavong, Y.; Santi, D. V. The dihydrofolate reductase domain of *Plasmodium falciparum* thymidylate synthase-dihydrofolate reductase. Gene synthesis, expression, and anti-folate-resistant mutants. *J. Biol. Chem.* **1993**, *268*, 21637–21644.
- Brobey, R. K. B.; Sano, G.; Itoh, F.; Aso, K.; Kimura, M.; Mitamura, T.; Horii, T. Recombinant *Plasmodium falciparum* dihydrofolate reductase-based in vitro screen for antifolate antimalarials. *Mol. Biochem. Parasitol.* **1996**, *81*, 225–237.
- Farber, P. M.; Becker, K.; Muller, S.; Schirmer, R. H.; Franklin, R. M. Molecular cloning and characterization of a putative glutathione reductase gene, the PFGR2 gene, from *Plasmodium falciparum*. *Eur. J. Biochem.* **1996**, *239*, 655–661.
- Sirawaraporn, W.; Sathitkul, T.; Sirawaraporn, R.; Yuthavong, Y.; Santi, D. V. Antifolate-resistant mutants of *Plasmodium falciparum* dihydrofolate reductase. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 1124–1129.
- Toyoda, T.; Brobey, R. K. B.; Sano, G.; Horii, T.; Tomioka, N.; Itai, A. Lead discovery of inhibitors of the dihydrofolate reductase domain of *Plasmodium falciparum* dihydrofolate reductase-thymidylate synthase. *Biochem. Biophys. Res. Commun.* **1997**, *235*, 515–519.
- Amin, N. M. Prophylaxis for malaria. Helping world travelers come home healthy. *Postgrad. Med.* **1992**, *92*, 161–168.
- Knighton, D. R.; Kan, C.; Howland, E.; Janson, C. A.; Hostomska, Z.; Welsh, K. M.; Matthews, D. A. Structure of and kinetic channelling in bifunctional dihydrofolate reductase-thymidylate synthase. *Struct. Biol.* **1994**, *1*, 186–194.
- Peterson, D. S.; Milhous, W. K.; Wellem, T. E. Molecular basis of differential resistance to cycloguanil and pyrimethamine in *Plasmodium falciparum* malaria. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 3018–3022.
- Douglas, K. T. Rational drug design in parasitology. *Parasitol. Today* **1994**, *10*, 389–392.
- Falco, E. A.; Russell, P. B.; Hitchings, G. H. 2,4-Diaminopyrimidines as antimalarials. I. 5-Aryloxy and 5-alkoxy derivatives. *J. Am. Chem. Soc.* **1951**, *73*, 3753–3758.
- Falco, E. A.; DuBreuil, S.; Hitchings, G. H. 2,4-Diaminopyrimidines as antimalarials. II. 5-Benzyl derivatives. *J. Am. Chem. Soc.* **1951**, *73*, 3758–3762.
- Russell, P. B.; Hitchings, G. H. 2,4-Diaminopyrimidines as antimalarials. III. 5-Aryl derivatives. *J. Am. Chem. Soc.* **1951**, *73*, 3763–3770.
- Read, M.; Hyde, J. E. Simple in vitro cultivation of the malaria parasite *Plasmodium falciparum* (erythrocytic stages) suitable for large-scale preparations. In *Protocols in Molecular Parasitology*; Hyde, J. E., Ed.; Humana Press: Totowa, NJ, 1993; pp 43–56.

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