CENTER FOR DRUG EVALUATION AND RESEARCH

APPLICATION NUMBER:

21-078

MICROBIOLOGY REVIEW

MICROBIOLOGY REVIEW
DIVISION OF SPECIAL PATHOGENS AND IMMUNOLOGIC DRUG PRODUCTS (HFD-590)

NDA #: 21-078	**REVIEWER**	: Shukal Bala
	CORRESPONDENCE DATE	: 12-29-98; 02-24-99 03-17-99; 03-31-99 05-17-99
	CDER RECEIPT DATE	: 12-29-98; 02-25-99 03-18-99; 04-01-99 05-18-99
	REVIEW ASSIGN DATE	: 01-05-99; 03-09-99 03-30-99; 04-08-99 05-24-99
	REVIEW COMPLETE DATE	: 05-24-99

SPONSOR: Glaxo Wellcome Research and Development
5 Moore Drive
P. O. Box 13398
Research Triangle Park, NC 27709

SUBMISSION REVIEWED: Original, BZ, NC, BI and BI

DRUG CATEGORY: Anti-parasitic

INDICATION: Treatment and prevention of *Plasmodium falciparum* malaria

DOSAGE FORM: Tablets for oral administration

PRODUCT NAMES:

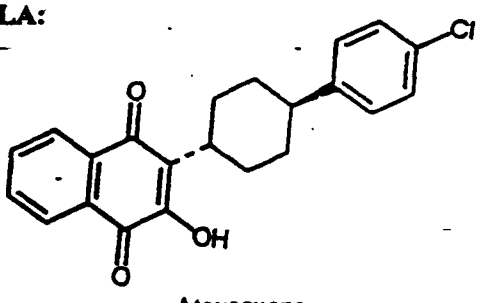
a. **PROPRIETARY:** Malarone

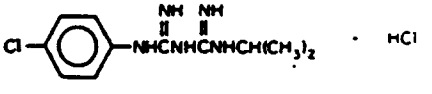
b. **NONPROPRIETARY:** Atovaquone and proguanil hydrochloride

c. **CHEMICAL:** Atovaquone: trans-2-[4-(4-chlorophenyl)cyclohexyl]-3-hydroxy-1,4-napthalenedione

Proguanil hydrochloride: 1-(4-chlorophenyl)-5-isopropyl-biguanide hydrochloride

STRUCTURAL FORMULA:





	Atovaquone	Proguanil hydrochloride
Molecular weight:	366.84	290.22
Empirical formula:	$C_{22}H_{19}ClO_3$	$C_{11}H_{16}ClN_5.HCl$

SUPPORTING DOCUMENTS: IND #
NDA # 20-500 and 20-259

BACKGROUND:

The subject of this NDA is Malarone, a combination of atovaquone and proguanil hydrochloride in the ratio of 2.5:1. Atovaquone is approved for the treatment and prophylaxis of *Pneumocystis carinii* pneumonitis in patients who are intolerant to trimethoprim/sulfamethoxazole. Proguanil, though not approved, has been used for the treatment of malaria. In this submission, the sponsor is seeking approval of Malarone (1) "for the **prophylaxis** of *Plasmodium falciparum* malaria infections, and (2) "for the **treatment** of acute, uncomplicated *P. falciparum* malaria.

For prophylaxis, the sponsor has proposed a combination of 250 mg atovaquone + 100 mg proguanil /day starting 1 or 2 days before entering a malaria endemic area and continuing for 7 days after return. For treatment, the sponsor has recommended that 1000 mg atovaquone + 400 mg proguanil be administered daily for 3 days.

Atovaquone, a highly protein bound compound, exhibits its antimalarial activity by inhibition of the mitochondrial respiratory chain at the cytochrome bc_1 complex (complex III). The half-life in humans is stated to be 2 to 3 days in adults and 1 to 2 days in children.

Proguanil is metabolized to cycloguanil and p-chlorophenylbiguanide (pCBG) in the presence of cytochrome p450 enzymes predominantly present in the liver. It is believed that these metabolites are not formed *in vitro*. Although the antimalarial activity of proguanil has been shown to be predominantly due to cycloguanil both proguanil and pCBG exhibit weak antimalarial activity against the erythrocytic stages of the parasite. Cycloguanil inhibits the activity of dihydrofolate reductase (DHFR), an enzyme essential for the formation of tetrahydrofolates. The half-life of proguanil and cycloguanil has been shown to be 15 to 17 hours in both adults and children.

Development of resistance by the *Plasmodium* parasite to atovaquone or proguanil alone is well known. The sponsor has stated that the combination of 2 drugs with different mechanism of actions is less likely to induce resistance. However, the potential for development of resistance against the combination of atovaquone + proguanil was not investigated.

Biology of the parasite

Over 100 species of *Plasmodium* have been shown to cause malaria in a wide variety of vertebrate hosts. The four species known to infect man are *P. falciparum*, *P. vivax*, *P. malaria*, and *P. ovale*.

The life cycle of the malaria parasite (*Plasmodium*) is divided between 2 hosts: mosquitoes (as a vector) and vertebrates (Figure 1). Infection in humans is initiated by the bite of infected mosquitoes and sporozoites are inoculated into the blood stream. The sporozoites are cleared from the blood within 1 – 2 hours, migrate to the liver and then invade the hepatocytes. The sporozoites develop into schizonts within a period of 5 to 15 days. During this phase the parasite undergoes nuclear division and merozoites (10,000 to 30,000) are formed which are released by the rupture of a liver schizont. This phase of the life cycle of *Plasmodium* is called **exoerythrocytic** or **liver schizogonic phase**. The merozoites invade the erythrocytes and progress through a series of developmental forms into mature erythrocytic schizonts. Each merozoite matures into a schizont, undergoes asexual multiplication, and forms 24 merozoites. The red blood cells rupture and the merozoites reinvade other erythrocytes. This part of the life cycle is called the **erythrocytic phase** and takes about 24 to 72 hours. Fever is associated with the rupture of infected red blood cells. Severe or fatal complications can occur at any time and are related to blockage of blood vessels in the internal organs such as brain, spleen, bone marrow, etc. Some merozoites mature into male and female gametocytes within the erythrocytes. The maturation of these gametes occurs once the infected blood is ingested by the mosquitoes. The gametes undergo fertilization within the vector and form a zygote. This is followed by the elongation of a zygote to form an ookinete in about 12 to 48 hours. The ookinetes invade the stomach of the mosquito and develop into oocysts. The oocyst enlarges forming over 10,000 sporozoites (in about a week after the formation of the oocyst). The sporozoites released by the rupture of the oocyst migrate to the salivary glands of the mosquitoes and are inoculated into humans the next time the mosquito feeds (i.e., during a blood meal).

In patients with *Plasmodium falciparum* infection, recurrence is generally thought to be due to recrudescence which is caused by residual erythrocytic forms; whereas in *P. vivax* and *P. ovale* relapse of an infection is considered to be due to dormant parasites in the liver which undergo development and release merozoites into circulation.

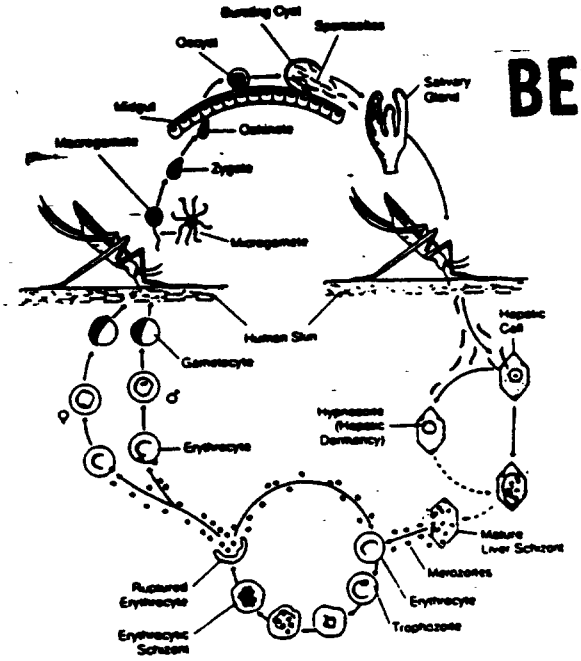


FIGURE 1 The life cycle of the malaria parasite. (Adapted from *Hospital Practice*, September 15, 1990, by permission of HP Publishing, New York)

SUMMARY:

The preclinical studies (submitted in the NDA or those reported in the literature) demonstrating anti-plasmodial activity of atovaquone and/or proguanil/ cycloguanil are summarized below:

A. Activity *in vitro*

Studies available to support the *in vitro* activity of atovaquone, proguanil or the combination of atovaquone + proguanil against different developmental forms of *Plasmodium* are as follows:

1. ***In vitro* activity against erythrocytic forms of *P. falciparum*:**
   (i) **Activity against laboratory strains:**
      (a) **Atovaquone**

In a study by Hudson *et al.*, 1991 (Drugs Exptl. Clin. Res. 17: 427), the *in vitro* activity of atovaquone was measured against the erythrocytic stages of *P. falciparum* by incubating different concentrations of the drug with 4 different strains of the parasite in RPMI 1640 medium containing 10% heat inactivated human plasma (type A) for 24 hours. The antiparasitic activity of the drug was then measured by incorporation of ^{3}H-hypoxanthine over an additional 18 hour incubation period (Desjardins *et al.*, 1979, Antimicrob. Agents Chemotherap. 16: 710). The raw data were not included in the publication. The calculated results shown in Table 1 are expressed as 50% inhibitory concentrations (IC_{50}) and are based on repeated testing against 4 isolates. The IC_{50} values against 4 different strains/clones varied from 0.7 to 4.3 nM (i.e., 0.26 to 1.58 ng/ml). The IC_{50} values of other antimalarials used for comparison were ≥ 12 fold higher (Table 1).

Table 1 In vitro antimalarial activity against various P. falciparum strains/clones (IC_{50} nM)[a]

Strain/Clone	T_{996}		FCW-1		H-1		D_6	
Country of origin	Thailand		Nigeria		Honduras		Sierra Leone	
566C80 (atovaquone)	1.3 ± 0.2	(41)	4.3 ± 0.5	(2)	3.7 ± 2.1	(9)	0.7 ± 0.2	(2)
58C80	2.5 ± 0.4	(16)	1.2 ± 0.3	(5)	13.0	(1)	0.4 ± 0.1	(4)
amodiaquine	6.7 ± 0.8	(3)	NT		8.3	(1)	NT	
mefloquine	54.2 ± 5.9	(16)	35.0 ± 7.5	(5)	61.9 ± 29.7	(3)	52.0 ± 17.3	(5)
chloroquine	73.6 ± 8.8	(27)	247.0 ± 148.2	(5)	106.0 ± 17.0	(4)	76.8 ± 21.0	(5)
pyrimethamine	162.5 ± 36.4	(19)	160.0 ± 25.6	(3)	1534.0 ± 1455.3	(5)	79.7 ± 41.7	(5)
quinine	284.0 ± 44.3	(14)	292.0 ± 71.5	(4)	460.0 ± 144.7	(3)	217.0 ± 68.4	(5)

[a] IC_{50}nM ± standard error; () = no. of tests, each carried out in duplicate. NT = not tested. T_{996} = clone derived from a Thai isolate (kindly donated by Dr D Walliker, Edinburgh) FCW-1 = Wellcome FCW-1/Nigeria strain H-1 = Honduras 1 strain. D_6 = Sierra Leone clone.

The sponsor submitted the representative raw data for the above study upon request from FDA. This information includes representative IC_{50} values from 32 different tests against 7 isolates (Table 2) along with estimates of ^3H hypoxanthine incorporated (expressed as cpm) for some of the tests. The results are expressed as IC_{50} and 95% confidence limit values. It is unclear what the 95% confidence limits represent. If these values are based on multiple testing and represent a range for 95% of the calculated IC_{50} values, then it is of note that for test numbers 18 and 23 in Table 2 have upper bound values of 29 and 55.7/18 nM (i.e., 10.6 and 20.4 ng/ml respectively).

Table 2

Atovaquone *in vitro* data:

Representative in vitro data from 32 different tests, using 7 different strains of P. falciparum, carried out from 1983-1991 (64 results: IC_{50})

Number	Date	Test No	Lab.Notebook Ref.	*P. falciparum* strain/clone	566C batch	IC_{50} nM	95% confidence limits
1	10/25/84	M183-188/84	ZEPA 84/19	T9/96	CD	1.1	not recorded
					CD	0.6	not recorded
2	2/24/86	MS21-30/86	ZEPA 86/7 93-96	T9/96	CE	0.6	not recorded
3	3/18/86	MS36-46/86	ZEPA 86/7 136	T9/96	CE	3	not recorded
4*	6/2/86	M82-86/86	ZEPA 86/7 154	T9/96	CE	0.16	0.22-0.14
5	6/12/86	M86-87/86	ZEPA 86/7 161	T9/96	CE	0.93	1.6-0.7
6	6/19/86	M77/86	ZEPA 86/7 167	T9/96	CE	1.2	1.5-0.9
7*	6/19/86	M84-85/86	ZEPA 86/7 170	T9/96	WA	0.3	0.6-0.1
					CE	0.6	0.9-0.3
8	7/3/86	M92-95/86	ZEPA 86/7 177	T9/96	CE	1.2	1.5-1.0
					WA	1.1	1.4-0.8
9	7/14/86	M102-105/86	ZEPA 86/7.184	T9/96	WA	1.22	1.5-0.99
					CE	0.86	1.54-0.5
10*	9/23/86	M128-129/86	ZEPA 86/12 63	T9/96	WA	0.2	0.4-0.8
					WB	0.1	0.3-0.03
					WC	0.2	0.4-0.8
					WD	0.2	0.3-0.14
11	2/6/87	M02/87	ZEPA 86/37 3	T9/96	WA	0.89	1.03-0.46
12	6/29/87	M06/87	ZEPA 86/3727	T9/96	CE	0.19	not recorded
					WA	0.44	not recorded
					WB	0.39	not recorded
					WC	0.38	not recorded
					WD	0.42	not recorded
					WE	0.26	not recorded
					WF	0.47	not recorded
13*	9/21/87	MST5/87	ZEPA 86/37 97	T9/96	WE	1.06	not recorded
14*	9/24/87	MST7/87	ZEPA 86/37 94	T9/96	WE	0.43	2.618- 0.07
15*	12/16/87	MR12/87	ZEPA 87/12 6	T9/96	WK	3.5	7.1-1.7
16	1/18/88	MST12/88	ZEPA 87/12 11	T9/96	WK	1.8	0.23-0.14
17	8/30/88	MS32-33/88	ZEPA 88/5 180,183	T9/96	WK	1	1.4-0.8
18	11/8/88	MSC6/88	ZEPA 88/8 142	T9/96	WK	17	29.0-10
19	1/18/89	MP1-6/89	ZEPA 88/11 140-141	T9/96	WK	2.8	3.5-2.0
					WK	2.7	4.0-1.9
					WK	2.7	3.3-2.3
					WK	2.7	3.2-2.2
					WK	3.2	4.1-2.5
20	2/20/89	MP7-14/89	ZEPA 89/2 40	T9/96	WK	1.5	2.0-1.5
					WK	2.5	3.4-1.9
					WK	2.3	3.0-1.8
					WK	2.5	4.8-1.3
					WK	1.9	2.8-1.3
					WK	3.4	5.1-2.0
					WK	2.3	3.0-1.8
21	7/17/90	913/90	ZEPA 89/06 125	T9/96	WK	1	1.11-0.95
22	5/9/91		ZEPA 90/2 93	T9/96	WK	0.74	0.87-0.63

Table 2 (continued)

Number	Date	Test No	Lab/Notebook Ref.	P. falciparum strain/clone	566C batch	IC_{50} nM	95% confidence limits
23	11/30/87		ZEPA 86/37	KI	WK	5.20	9.7-2.8
					WK	3.1	55.7-0.17
					WK	4.7	10.0-1.2
24	6/2/86	M82-66	ZEPA 85/7 154	HI	CE	2.5	4.9-1.2
25	6/29/87		ZEPA 86/37 27	HI	CE	1.13	not recorded
					WA	0.96	not recorded
					WB	1.63	not recorded
					WC	1.75	not recorded
					WD	1.53	not recorded
					WE	1.44	not recorded
					WF	2	not recorded
26*	2/18/86	MS21-30/86	ZEPA 85/7 94	D6	CE	0.41	1.12-0.16
27	3/18/86	MS35-46/86	ZEPA 85/7138	D6	CE	0.9	1.14-0.68
28	9/20/83	MZ20-Z21/83	ZEPA 83/21 39	FCW-1	CC	1.3	1.66-1.06
						2	2.3-1.79
29	2/18/86	MS21-3086	ZEPA 85/7 86	FCW-1	CE	0.44	0.49-0.35
30	3/18/86	MS35-46/86	ZEPA 85/7 136	W2	CE	2.73	2.39-0.327
31	2/1/90	R1-R4/90	ZEPA 89/07 14	W2	WK	3	4.0-2.0
32	3/18/86	MS35-46/86	ZEPA 85/7 140	CAMP	CE	2.6	3.54-1.99

* cpm data provided separately

In another study (BUAC/88/CI), the activity of 3 different batches of atovaquone was measured using the FCW-1 strain of *P. falciparum* by incorporation of ^3H hypoxanthine. The results in Table 3 show the IC_{50} values to be in the range of 0.55 – 1.0 ng/ml. The IC_{99} values were 61 – 969 fold higher than the IC_{50} values.

Table 3

P.falciparum

Batch		IC_{50}	SD	n	IC_{99}	SD	n	Est IC_{50}	Est IC_{99}
CE	x 10^{-9} M	1.5	1.03	9	90.5	145	5		
	ng/ml	0.55	0.37	9	33	53	5	1	100
RA	x 10^{-9} M	2.8	2.2	17	318	466	14		
	ng/ml	1.0	0.81	17	117	171	14	2	300
WK (Q4M)	x 10^{-9} M	2.23	0.58	8	2160	2510	7		
	ng/ml	0.82	0.21	8	792	920	7	1.5	1,800

In another study, erythrocytic forms of *P. falciparum* (FCR3F86 strain) were cultured in the presence of atovaquone and incubated for 48 hours (Murphy and Lang-Unnasch, 1999, Antimicrobial Agents Chemother. 43: 651). The activity of the drug was measured by the incorporation of ^{3}H hypoxanthine. The IC_{50} values based on repeat testing were 2.1 ± 0.2 nM (i.e., 0.77 ± 0.07 ng/ml).

(b) Proguanil

In a study by Watkins *et al.*, 1984 (Ann. Trop. Med. Parasitol. 78: 273), the *in vitro* activity of proguanil and its metabolites (cycloguanil and p-chlorophenylbiguanide) was measured in normal medium (RPMI 1640) against 10 different strains of *P. falciparum* by incorporation of ^{3}H-hypoxanthine (Desjardins *et al.*, 1979, Antimicrob. Agents Chemother. 16: 710). Seven of these strains were collected from Kenyan children and 3 were from South East Asia (SEA). One of the strains (FVO) from SEA was known to be sensitive to pyrimethamine and the remaining 2 (Camp and Smith) were resistant to pyrimethamine. Different dilutions of the drugs were incubated with an equal volume of infected erythrocytes (0.4%) in RPMI 1640 containing 10% human serum. The experiments were repeated 3 times over a period of 3 weeks. The raw data were not included in the report.

The results shown in Table 4 indicate that cycloguanil IC_{50} values ranged from 17.6 to 5108 nM (4.4 to 1287.2 ng/ml); whereas that of proguanil (2422 to 19243 nM i.e., 615.2 to 4887.7 ng/ml) and p-chlorophenylbiguanide (11376 to 40662 nM i.e., 2411.7 to 8620.3 ng/ml) were about 4 to 472 fold higher. The FVO strain was stated to be resistant to cycloguanil. However, the basis for establishing the cycloguanil threshold of resistance is not specified. The sensitivity/resistance to cycloguanil did not correlate with sensitivity/resistance to pyrimethamine (another DHFR inhibitor).

TABLE 4

Proguanil, cycloguanil, p-chlorophenylbiguanide, pyrimethamine and chloroquine activity in vitro *(50% inhibitory concentration, nmol l^{-1}, mean ± S.E.) against seven Kenyan and three South East Asian strains of* Plasmodium falciparum

Plasmodium falciparum *strain*	*Pyrimethamine*	*Cycloguanil*	*Proguanil*	*p-chlorophenyl-biguanide*	*Chloroquine*
FVO	145·7 ± 20·9	5108 ± 340	19243 ± 3559 (3·8)	22913 ± 2567 (4·5)	209·5 ± 20·6
Camp	805·4 ± 72·4	78·7 ± 25·7	12820 ± 2723 (12·9)	N.T.*	30·8 ± 10·4
Smith	6948 ± 1005	2215 ± 1108	13092 ± 3161 (5·9)	40662 ± 5887 (15·4)	122·9 ± 31·0
M24	38·9 ± 3·3	17·6 ± 6·6	8315 ± 875 (47·1)	N.T.	24·4 ± 6·4
M25	60·0 ± 29·8	31·5 ± 8·0	7050 ± 4918 (223·9)	N.T.	30·0 ± 11·2
M32	55·9 ± 10·8	36·0 ± 1·6	2422 ± 540 (67·2)	N.T.	35·1 ± 14·5
K33	65·9 ± 19·8	31·8 ± 3·9	12075 ± 1214 (379·7)	11376 ± 2000 (35·7)	27·6 ± 3·2
K41	47·0 ± 9·3	30·4 ± 7·1	7716 ± 4812 (253·8)	N.T.	21·4 ± 4·8
K34	7237 ± 4769	761·9 ± 165	7346 ± 1454 (9·6)	N.T.	48·9 ± 3·3
K39	5122 ± 1166	715·9 ± 250	13896 ± 1939 (19·4)	N.T.	31·0 ± 3·6

*N.T. = not tested.

(c) Activity of a combination of atovaquone + proguanil

In a study by Canfield *et al.*, 1995 (Experimental Parasitol. 80: 373), the *in vitro* activity of a combination of atovaquone + proguanil (at different concentrations and in different proportions) was tested against five strains/clones of *P. falciparum*. The characteristics of the strains/clones used in the study are as follows:

1. T9/96, a drug-sensitive(*) Thai clone
2. R_5, a hydroxynaphthoquinone resistant clone derived from T9/96 clone
3. W-2, a multidrug resistant clone (obtained from Walter Reed)
4. D-6, a mefloquine resistant(*) clone (obtained from Walter Reed)
5. C2B, a hydroxy naphthoquinone resistant isolate obtained from a Thai patient (after an R1-type recrudescence occurred during the phase 2 atovaquone clinical trial. The IC_{50} for atovaquone of the C2B isolate was 95 times higher than that of the pretreatment isolate from the same patient thereby suggesting clinically relevant atovaquone resistance).

[*Please note that although clone T9/96 was stated to be drug sensitive and D6 drug resistant the IC_{50} values against various antimalarials (mefloquine, chloroquine, pyrimethamine, and quinine) were shown to be similar by another group of workers, see Table 1, page 4]

Activity of the drugs was measured by incorporation of radiolabeled hypoxanthine using a semi-automated microdilution technique (Desjardins *et al.*, 1979, Antimicrobial. Agents Chemotherapy 16: 710). In the initial experiment the observations (Table 5) were based upon 43 hours of incubation and indicate a synergistic interaction between atovaquone and cycloguanil using the R5 clone. Against the T9/96 clone, the interaction varied from synergy to weak antagonism depending on the statistical method of analysis. In another experiment, cultures of the 3 laboratory strains (W2, D6, and C2B) were incubated with the drug(s) for 72 hours. The interaction between atovaquone and proguanil was shown to be synergistic against all 3 strains (Table 6). Interaction between atovaquone and cycloguanil (metabolite of proguanil) was also shown to be synergistic against w-2 and C2B strains. However, the activity against the D-6 strain varied from weak synergism to weak antagonism.

The authors have stated that the *in vitro* ratios of the drugs used showed wide variation in activity against various strains. For example, the optimum ratio of atovaquone:proguanil was found to be 4000:1 and 0.2:1 against D-6 and C2B isolates respectively. Therefore, the *in vitro* data on the drug combinations were not considered to be useful for determining the ratios to be used in humans. Also, it should be noted that this study was based on testing of drug activity against 3 to 5 isolates only.

TABLE 5

Interactions of Atovaquone with Other Antimalarial Drugs against *Plasmodium falciparum in Vitro*: Preliminary Studies

Drug	SUM FIC[a]:T9/96[b]	Chequerboard:T9/96[b]	Chequerboard:R_5[b]
Quinine	2.2[c] Antagonism	Weak antagonism	Antagonism
	1.5[d] Antagonism	—	—
Chloroquine	1.7[e] Antagonism	—	Synergy
	0.17[e] Synergy	—	—
Mefloquine	0.5[c] Synergy	Weak antagonism	Weak antagonism
Halofantrine	1.4[c] Antagonism	Antagonism	—
Primaquine	0.7[c] Synergy	—	—
Tetracycline	0.75[f] Synergy	Synergy	—
Doxycycline	1.0[c] Addition	—	Synergy
Artemisinin	3.0[c] Antagonism	Synergy	Synergy
Cycloguanil	0.5[c] Synergy	Weak antagonism	Synergy
Pyrimethamine	—	Synergy	Addition
Clopidol	0.4[d] Synergy	—	—
4-Pyridone	0.3[b] Synergy	Synergy	Synergy

[a] FIC values <1 = syngerism; FIC values >1 = antagonism; FIC values of 1 = addition.
[b] T9/96 is a drug sensitive strain; R_5 is a hydroxynaphthoquinone resistant clone.
[c] Ratio 10:1.
[d] Ratio 100:1; 1000:1.
[e] Ratio 7000:1.
[f] Ratio 0:1.

TABLE 6

Interactions of Atovaquone with Other Antimalarial Drugs against *Plasmodium falciparum in Vitro*: Detailed Studies

Drug	Strain[a]	I[b]	Interaction
Chloroquine	W-2	−1.84	Antagonism
	D-6	−1.40	Antagonism
Mefloquine	W-2	−1.19	Antagonism
Quinine	W-2	−1.36	Antagonism
Primaquine	W-2	−0.79	Weak antagonism
Ciprofloxacin	W-2	−1.22	Antagonism
Norfloxacin	W-2	+1.02	Synergy
Tetracycline	W-2	+1.27	Synergy
	D-6	+1.11	Synergy
	C2B	+0.02	Addition
	C2B	−0.08	Addition
Artesunate	W-2	−0.18	Weak antagonism
PM443[c]	W-2	−1.28	Antagonism
Allopurinol[d]	W-2	+1.14	Synergy
	D-6	+0.43	Weak synergy
Dapsone[d]	W-2	−0.39	Weak antagonism
Sulphamethoxazole[d]	W-2	+2.75	Synergy
Cycloguanil[e]	W-2	+2.21	Synergy
	D-6	+0.13	Weak synergy
	D-6	−0.73	Weak antagonism
	C2B	+1.66	Synergy
Proguanil[e]	W-2	+2.88	Synergy
	W-2	+2.43	Synergy
	D-6	+2.56	Synergy
	C2B	+2.56	Synergy
PS-15[d,e]	W-2	+1.77	Synergy
	W-2	+0.65	Weak synergy
	W-2	+1.97	Synergy
	D-6	−0.74	Weak antagonism
WR99210[d,f]	W-2	+0.02	Addition
Pyrimethamine[d]	W-2	+0.36	Weak synergy
	C2B	−0.48	Weak antagonism
Trimethoprim[d]	W-2	+1.27	Synergy
	C2B	+0.58	Weak synergy
Clopidol[d]	W-2	+2.38	Synergy
	W-2	+2.65	Synergy
	D-6	+0.73	Weak synergy

[a] W-2, multidrug resistant; D-6, mefloquine resistant; C2B; hydroxynaphthoquinone resistant.
[b] Negative I, antagonism; zero I, addition; positive I, synergism.
[c] Jefford (1991) 1, 2, 4-trioxane.
[d] Measured using RPMI 1640 with low physiological levels of folate and p-aminobenzoate.
[e] Canfield-*et al.* (1993) biguanide.
[f] WRAIR triazine (Canfield 1986).

(ii) Activity of atovaquone, cycloguanil or proguanil against fresh clinical isolates *in vitro*

Pretreatment blood samples were collected from 12 patients in Thailand with *P. falciparum* infection for *in vitro* culture and determination of drug susceptibility (Looareesuwan *et al.*, 1996, Am. J. Trop. Med. Hyg. 54: 62). The variability in IC_{50} values was not described in the publication. The authors stated that the mean IC_{50} value for atovaquone against this group of isolates was 3.3 ng/ml i.e., 8.99 nM. The authors have also stated that these isolates were resistant to chloroquine. However, the actual data were not included in the report.

In another study by Gay *et al.*, 1997 (Am. J. Trop. Med. Hyg. 56: 315), blood samples were collected from 142 subjects with *P. falciparum* infection [108 from Central and West Africa (nonsevere patients treated with halofantrine, 1500 mg/kg total dose or severe malaria patients treated with quinine 24 mg/kg/day for 5 days), 34 from Asia of which 32 were from Philippines (uncomplicated malaria treated with atovaquone + proguanil or chloroquine + sulfadoxine-pyrimethamine), and 1 each from Laos and Myanmar (previous treatment not specified)]. The parasites isolated were tested within 4 – 14 hours of sample collection for *in vitro* susceptibility against various antimalarial agents. Different concentrations of the drugs were incubated with the parasite in RPMI 1640 medium containing 10% heat inactivated serum by the method of Bustos *et al.*, 1991-1993 (Bull. World Health Organization 72: 729). Results, expressed as median IC_{50} values (90th percentile) for 62 African and 34 Asian strains, were 1.6 (5.3) nM [0.587 (1.9) ng/ml] and 1 (6.0) nM [0.37 (2.2) ng/ml] respectively (Table 7). The full range of IC_{50} values were not included in the publication. The authors have stated that 18 of the 32 patients from Philippines received Malarone (1000 mg atovaquone + 400 mg proguanil, daily for 3 days) and were cured. The pretreatment IC_{50} values in the 3/18 patients were stated to be 6.1, 9.1 and 9.6 nM (i.e., 2.2, 3.3 and 3.5 ng/ml).

TABLE 7

In vitro drug sensitivity test results for *Plasmodium falciparum* strains from Africa and Asia*

	Africa			Asia			
Drug	n	Median IC_{50}	90th percentile	n	Median IC_{50}	90th percentile	P
CQ	108	34.5	137.0	31	54	660	0.008
QN	107	81.0	186.0	30	132	1,893	0.11
MQ	108	4.3	20.2	30	7.3	119.9	0.09
HF	108	1.3	4.8	31	2.4	21.7	0.0006
AI	108	4.0	15.1	34	7.3	105.3	0.036
AM	108	2.6	10.9	34	6.1	120.1	0.08
AE	92	2.4	9.3	28	3.2	61.7	0.07
AU	108	1.0	3.7	34	1.3	21.9	0.45
AQ	62	1.6	5.3	34	1.0	6.0	0.14

* IC_{50} = 50% inhibitory concentration (nmol/L). CQ = chloroquine: resistance threshold (R) = 100 nmol/L; QN = quinine: R = 350 nmol/L; MQ = mefloquine: R = 20.0 nmol/L; HF = halofantrine: R = 4.0 nmol/L; AI = artemisinin: R = not determined; AM = artemether: R = not determined; AE = arteether: R = not determined; AU = artesunate: R = not determined; AQ = atovaquone: R = not determined.

In another study by Basco *et al.*, 1995 (Am. J. Trop. Med. Hyg. 53: 388), 61 fresh clinical isolates obtained from travelers returning from Africa to France and 3 laboratory isolates [2 chloroquine susceptible (L-3/Coted'Ivoire and L-16/Sierra Leone) and 1 chloroquine resistant (FCM 29/Cameroon)] were tested for *in vitro* susceptibility by the method of Le Bras and Deloron, 1983 (Am. J. Trop. Med. Hyg. 32: 447) against atovaquone, and other antimalarial drugs. The cultures were incubated for 42 hours in RPMI 1640 containing 10% human serum. Activity of cycloguanil was, however, measured in RPMI SP241 (folate- and ρ-aminobenzoic acid free) medium. The antimalarial activity was measured by incorporation of ^{3}H-hypoxanthine at 18 hours of culture. The atovaquone IC_{50} for the chloroquine susceptible (IC_{50} < 100 nM) and resistant (IC_{50} > 100 nM) strains and the fresh

clinical isolates were similar (Tables 8 and 9; $p > 0.05$). Also, there was no correlation between *in vitro* susceptibility to atovaquone and other antimalarial agents ($p > 0.05$, see Table 10). Although cycloguanil was also used for testing in this study, the data were not included in the publication. The authors have stated in the footnote to Table 9 that 23/28 chloroquine susceptible and 9/20 chloroquine resistant isolates were susceptible to cycloguanil ($IC_{50} < 50$ nM) *in vitro*.

Table 8

Strains/Clones	Chloroquine susceptibility	Mean IC_{50} (Range) nM
L-3	+	0.978 (0.73 – 1.375)
L-16	+	0.680 (0.5 – 1.029)
FCM 29	-	1.76 (1.38 – 2.66)

TABLE 9

In vitro activity of atovaquone and other antimalarial drugs against African isolates of *Plasmodium falciparum*

Drug*	Chloroquine-susceptible (n = 35)		Chloroquine-resistant (n = 26)	
	Mean IC_{50} (nM)†	95% confidence limits	Mean IC_{50} (nM)†	95% confidence limits
Chloroquine	25.1	20.3–31.0	195	163–236
Quinine	105	86.3–129	182	153–216
Mefloquine	12.3	9.02–16.7	5.24	4.28–6.40
Halofantrine	2.04	1.53–2.72	0.787	0.658–0.944
Artemether	4.84	3.78–6.18	3.34	2.73–4.08
Atovaquone	0.889	0.740–1.07	0.906	0.759–1.08

* Twenty-three of 28 chloroquine-susceptible isolates and nine of 20 chloroquine-resistant isolates were susceptible in vitro ($IC_{50} < 50$ nM) to cycloguanil (active metabolite of proguanil).
† IC_{50} = geometric mean 50% inhibitory concentration.

TABLE 10

Correlation between the *in vitro* responses of various antimalarial drugs against African isolates of *Plasmodium falciparum**

Drug pairs		r	P
Chloroquine	Quinine	0.443	<0.01
Chloroquine	Mefloquine	−0.520	<0.01
Chloroquine	Halofantrine	−0.601	<0.01
Chloroquine	Artemether	−0.310	0.016
Quinine	Mefloquine	0.157	NS
Quinine	Halofantrine	0.098	NS
Quinine	Artemether	0.236	NS
Mefloquine	Halofantrine	0.943	<0.01
Cycloguanil	Pyrimethamine	0.959	<0.01
Atovaquone	Chloroquine	0.010	NS
Atovaquone	Quinine	0.129	NS
Atovaquone	Mefloquine	−0.143	NS
Atovaquone	Halofantrine	−0.110	NS
Atovaquone	Artemether	−0.096	NS
Atovaquone	Cycloguanil	0.052	NS
Atovaquone	Pyrimethamine	0.128	NS

* The correlation coefficient (r) was calculated by linear regression analysis of the logarithmic 50% inhibitory concentration values. Degrees of freedom = 60 (47 for cycloguanil and pyrimethamine). NS = not significant ($P > 0.05$).

In a study by Zalis *et al.*, 1998 (Am. J. Trop. Med. Hyg. 58: 630), chloroquine resistant (W2), chloroquine sensitive (D6) and 26 Brazilian isolates were tested for atovaquone susceptibility *in vitro* by incorporation of ^{3}H hypoxanthine. Results show that the IC_{50} values for atovaquone varied from 0.04 – 2.7 nM (i.e., 0.015 to 0.99 ng/ml). It was also shown that several different alleles of merozoite surface antigen (MSA)-1 and MSA-2 were expressed among the Brazilian isolates. The presence of different alleles of MSA-1 and MSA-2 coupled with DNA fingerprinting indicates a mixed infection in the Brazilian population. It also demonstrates the activity of atovaquone against different clones/strains.

In a study by Ringwald *et al.*, 1996 (Am. J. Trop. Med. Hyg. 55: 254), 86 fresh clinical isolates of *P. falciparum* obtained from symptomatic patients from Yaounde, Cameroon were cultured with different concentrations of cycloguanil in RPMI 1640 medium containing 10% human serum and low concentrations of folic acid and ρ-aminobenzoic acid (Basco *et al.*, 1995, Am. J. Trop. Med. Hyg. 53: 388). The cultures were processed as described above. The results in Table 11 indicate that the IC_{50} values are > 100-fold lower for the chloroquine sensitive strains as compared to chloroquine resistant strains. Overall the IC_{50} values ranged from 6.73 to 1360 nM (i.e., 1.7 to 342.7 ng/ml).

TABLE 11

In vitro susceptibility of Cameroonian isolates of *Plasmodium falciparum* to pyrimethamine and cycloguanil

	In vitro response (nM)*					
	Susceptible†		Intermediate†		Resistant†	
Drug	IC_{50}	95% confidence limits	IC_{50}	Range	IC_{50}	95% confidence limits
Pyrimethamine	16.3 (n = 25)	11.8–22.7	409 (n = 2)	179–936	7,710 (n = 16)	6,110–9,750
Cycloguanil	8.79 (n = 25)	6.73–11.5	144 (n = 4)	56.2–366	1,030 (n = 14)	778–1,360

* Values are the geometric mean 50% inhibitory concentration (IC_{50}).
† Based on the previous study on African isolates;[11] in vitro response was defined as susceptible (IC_{50} < 100 nM for pyrimethamine, < 50 nM for cycloguanil), intermediate (IC_{50} 100–2,000 nM for pyrimethamine, 50–500 nM for cycloguanil), and resistant (IC_{50} > 2,000 nM for pyrimethamine, > 500 nM for cycloguanil)

In a study by Watkins *et al.*, 1987 (Am. J. Trop. Med. Hyg. 37: 445), the activity of cycloguanil was measured against 26 isolates from subjects in Kenya. The parasitized erythrocytes were cultured in the presence or absence of drug in RPMI 1640 medium free of ρ-aminobenzoic aminobenzoic acid and folic acid. Cultures were incubated for 66 hours and ^{3}H hypoxanthine added at 48 hours of culture. The results in Table 12 show about 15–fold increase in geometric mean IC_{50} values for the pyrimethamine resistant (14 nM i.e., 3.5 ng/ml) as compared to pyrimethamine-sensitive (0.916 nM i.e., 0.23 ng/ml) isolates.

TABLE 12

Geometric mean ID_{50} of five drugs against 10 pyrimethamine-sensitive and 16 pyrimethamine-resistant P. falciparum *isolates from Jilore, Kenya*

	ID_{50} mol/l (mean ± SD)		
	Pyrimethamine		
Drug	Sensitive	Resistant	*P* value of difference*
Pyrimethamine	$2.77 \pm 1.98 \times 10^{-10}$	$2.09 \pm 1.64 \times 10^{-7}$	$P < 0.01$
Cycloguanil	$9.16 \pm 1.41 \times 10^{-10}$	$1.40 \pm 1.58 \times 10^{-8}$	$P < 0.01$
Chlorcycloguanil	$2.36 \pm 1.99 \times 10^{-10}$	$1.82 \pm 2.13 \times 10^{-9}$	$P < 0.01$
MB 35769	$1.16 \pm 6.92 \times 10^{-9}$	$7.06 \pm 2.24 \times 10^{-9}$	$P > 0.05$
Chloroquine	$1.87 \pm 3.06 \times 10^{-8}$	$1.79 \pm 2.75 \times 10^{-8}$	$P > 0.05$

* By Student's *t*-test.

In another study by Khan *et al.*, 1997 (Trans. Roy. Soc. Trop. Med. Hyg. 91: 456), the *in vitro* susceptibility of 21 isolates from subjects in Kenya was measured against cycloguanil. The antiproliferative effect of the drug was measured by incorporation of ^{3}H hypoxanthine in medium free of ρ-aminobenzoic acid and folic acid. However, the total time of incubation was not specified. The results in Table 13 show that the cycloguanil IC_{50} values varied from 0.4 to 22.6 nM (i.e., 0.1 to 5.7 ng/ml). The mean IC_{50} values were higher for the pyrimethamine-resistant (14.4 nM i.e., 3.6 ng/ml) as compared to the pyrimethamine-sensitive (1.7 nM i.e., 0.4 ng/ml) isolates.

Table 13. Point mutations and sensitivity *in vitro* to pyrimethamine, cycloguanil and chlorcycloguanil of Kenyan isolates of *Plasmodium falciparum*

Isolates	Amino acids[a] 51	59	108	ID_{50}[b] (nM) Pyrimethamine	Cycloguanil	Chlorcycloguanil
M24	N	C	S	2·3±1·1	1·9±0·5	0·8±0·2
JP62	N	C	S	1·7±0·6	2·5±0·5	0·4±0·5
K67	N	C	S	0·3±0·2	0·9±0·1	0·2±0·2
S136	N	C	S	5·1±0·4	1·4±0·1	0·9±0·2
ENT22	N	C	S	3·6	1·7	0·5
D7/22	N	R	N	419·0±100	13·9±0·6	4·0±0·6
JP78	N	R	N	188·0±45	9·9±1·9	3·2±2·2
JP17/A	N	R	N	184·0±34	18·5±3·0	5·7±0·7
ENT7	N	R	N	532·0±176	21·6±1·9	5·4±2·5
ENT24	N	R	N	583·0±112	17·2±4·0	4·5±1·1
ENT11	N	R	N	349·0±150	11·0±5·5	3·4±1·0
JP1	N	R	N	227·0±80	7·9±1·6	1·4±1·1
ENT37	N	R	N	785·0±113	22·2±0·6	6·5±0·6
S104	N	R	N	347·0±68	12·2±2·2	4·2±2·1
K39	I	C	N	581·0±210	19·0±2·5	4·1±1·1
S158	I	C	N	362·0±163	15·2±8·4	2·9±1·9
JP119	I	C	N	246·0±50	11·7±4·2	3·2±0·2
KIL9	I	C	N	649·0±123	14·8±3·1	4·5±1·9
ENT36	I	R	N	296·0±128	13·4±2·7	3·6±2·0
ENT41	N/I	C/R	S/N	290/1·2±1·7	22·6/0·4±0·1	ND/0·1±0·01
ENT30	N/I	C/R	S/N	337/86	22·0/ND[c]	8·6/ND[c]

[a] See Table 2, footnote c, for explanation of the abbreviations.
[b] Concentration producing 50% inhibition of parasite growth *in vitro*.
[c] Not determined.

In the study by Nzila-Mounda *et al.*, 1998 (Antimicrob. Agents Chemother. 42: 164), 69 Kenyan *P. falciparum* isolates (erythrocytic forms) were cultured medium free of ρ-aminobenzoic acid and folic acid in the presence of cycloguanil and incubated for 48 hours. The IC_{50} values for cycloguanil varied from 0.03 – 37.26 nM (0.008 – 13.7 ng/ml). The analysis of dihydrofolate reductase (DHFR) phenotype by polymerase chain reaction showed that 37 of the isolates had different DHFR alleles, demonstrating the susceptibility of mixed isolates to cycloguanil.

In study MALB2001 (conducted by the sponsor to assess the prophylactic activity of Malarone), Kenyan patients (immune subjects from the endemic area) were administered Malarone (1000 mg atovaquone + 400 mg proguanil) for 3 days (radical cure) followed by prophylaxis with high (500 mg atovaquone/200 mg proguanil) or low (250 atovaquone/100 mg proguanil) dose Malarone for 10 weeks. The subjects were followed for development of parasitemia for 4 weeks after discontinuation of prophylaxis. After the 3-day radical cure phase all of the subjects in the low and high dose group were negative for parasitemia. The *in vitro* susceptibility of the erythrocytic forms of the parasite was measured at the time of recrudescence of parasitemia (week 5 – 14) in 8 subjects. Of these, 7 were on placebo and one was on low dose therapy (patient 082; parasitemia 16 days after discontinuation of prophylaxis) during the prophylactic phase. The atovaquone, cycloguanil and proguanil IC_{50} values (measured by incorporation of ^3H hypoxanthine) shown in Table 14 were in the range of 0.82 to 1.25 (0.3 – 0.46 ng/ml), 9.0 to 107.6 (2.3 – 27.1 ng/ml) and 3239 to 13233 (822.7 – 3361.2 ng/ml) nM respectively.

Based on the historical data, the sponsor stated that 6/8 isolates were resistant to chloroquine (IC_{50} values > 45 nM). However, the basis for selection of 45 nM as the chloroquine resistance threshold was not specified. The sponsor was asked to define and justify their process for establishing chloroquine resistance. The sponsor stated that the *in vitro* susceptibility testing was performed by Dr. Frank Klotz of the US Army Research Unit in Kenya and is based on unpublished data by a variety of investigators at the Walter Reed Army Institute of Research in Washington D.C. However, these data were not provided. It should be noted that in the study by Basco *et al.*, 1995 (Am. J. Trop. Med. Hyg. 53: 388) the IC_{50} values for chloroquine susceptible strains were in the range of 20.3 - 31.0 nM (geometric mean 25.1 nM) and 163 - 236 nM (geometric mean 195 nM) for chloroquine resistant strains. As in the previous study 100 nM was considered to be the threshold for chloroquine resistance (page 10).

Microscopic examination

Studies by Yeo *et al.*, 1997 (Acta Tropica 67: 207; Parasitol. Research 83: 489; and Biochemical Pharmacology 53: 943) measured *in vitro* activity by microscopic examination of Giemsa stained smears after the parasites were exposed to the drug for 48 hours. The inhibition of parasite reinvasion was used as the criteria of inhibition. The minimum inhibitory concentration (MIC) for atovaquone, cycloguanil, and proguanil for *P. falciparum* isolates (K1: resistant to pyrimethamine and chloroquine or FC27) synchronized with 5% sorbitol are summarized in Table 15.

Table 14

Subject Number	Radical Cure (3 days) / Treatment (Malarone)	IC_{50} Values (nM)			
		Atovaquone	Proguanil	Cycloguanil	Chloroquine
002 + / -	14	0.82	10406	59.0	61.9
017 + / -	5	1.96	13233	14.9	119.3
021 + / -	5	0.98	4687	107.6	82.6
062 + / low dose	12	1.69	3239	22.2	48.2
100 + / -	5	2.34	11392	9.0	6.0
112 + / -	5	0.60	4318	90.3	11.9
156 + / -	6	2.51	8629	28.5	107.8
196 + / -	5	1.25	5927	76.4	71.1
Mean		1.53	7729	51.0	63.6
Median		1.47	7278	43.7	66.5
Range		0.60–2.51	3239–13233	9.0–107.6	6.0–119.3
Historical Mean		1.73	10405	23.8	30.9
Cited Median Data[a]		1.61			34.4
Cited Geometric Mean Data[b]		0.90			25.10

[a] Gay F, Bustos D, Traore B, *et al. In vitro* response of *Plasmodium falciparum* to atovaquone and correlation with other antimalarials: comparison between African and Asian strains. Am J Trop Med Hyg 1997;56:315–7.

[b] Basco LK, Ramiliarisoa O and Le Bras J. *In vitro* activity of atovaquone against the African isolates and clones of *Plasmodium falciparum*. Am J Trop Med Hyg 1995;53:388–91.

+ = Radical cure for 3 days
- = Placebo for 10 weeks

Low dose = Malarone (atovaquone 250 mg/proguanil 100 mg)

Table 15

Reference	*P. falciparum* Clone	MIC (ng/ml) Atovaquone	Cycloguanil	Proguanil
Yeo *et al.*, 1997, Acta Tropica 67: 207	K1	15 ± 15	20 ± 12	8681 ± 3472
Yeo *et al.*, 1997, Parasitol Research 83: 489	K1	15 ± 3	ND	ND
	FC27	20 ± 5	ND	ND
Yeo *et al.*, 1997, Biochemical Pharmacology 53: 943	K1	14.7	20	7594.6

2. *In vitro* activity against hepatic forms of *Plasmodium*:
(a) Atovaquone

In a study by Davies *et al.*, 1993 (Parasitology 106: 1), about 1.5 – 2 x 10^4 sporozoites of *P. berghei* ANKA strain (isolated from the salivary glands of the mosquitoes *Anopheles stephensi*, 16 – 28 days after feeding on an infected mouse) were added to confluent layers of irradiated HepG2 cells in MEM containing 10% fetal calf serum. The cultures were incubated for 3 hours, and the medium changed before addition of the drug. After 48-hours of incubation with the drug, the cultures were fixed and stained with Giemsa and the number of schizonts/well counted. The results in Figure 2 and Table 16 indicate that atovaquone at a concentration of ≥ 13.6 nM (i.e., 4.99 ng/ml) was effective in inhibiting the development of schizont stage from the sporozoite stage *in vitro*. The IC_{50} was calculated to be 1.85 x 10^{-9} M (i.e., 0.68 ng/ml). Pyrimethamine was also effective in inhibiting the parasite and the IC_{50} value was calculated to be 1.95 x 10^{-8} M (Table 17 and Figure 3). Cytotoxicity data for the hepatic cells were not provided but the authors stated that no toxic effects were observed by light microscopy at the highest drug concentrations tested.

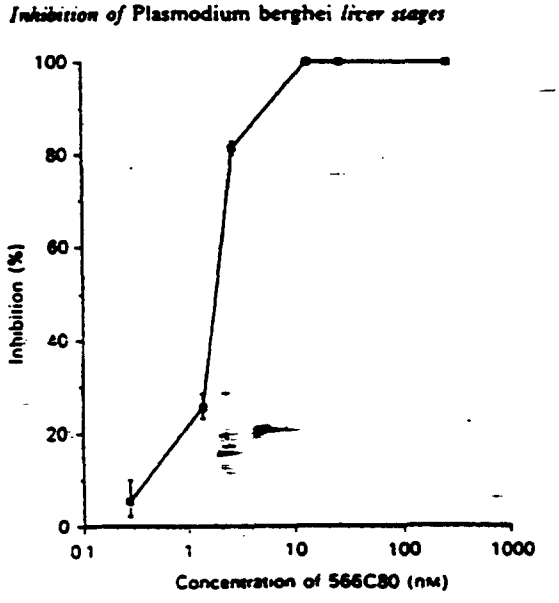


Fig. 2 Concentration-dependent effect of 566C80 on the numbers of *Plasmodium berghei* EE stages developing in HepG2 cells, expressed as percentage inhibition compared to control preparations receiving medium without drug. EE cultures were treated with drug 3 h after sporozoite inoculation and were examined 45 h later. Each drug concentration was tested on 4 replicates. Error bars are standard errors.

Table 16 Effect of 566C80 on the numbers of *Plasmodium berghei* EE stages developing in cultured HepG2 cells

Concentration of 566C80 (nM)	Mean no. of EE forms ±S.E. (n = 4)	Ethanol
0	586·25 ± 12·07	
0·27	554·25 ± 19·85*	- 1 %
1·36	433·75 ± 12·69†	
2·73	108·75 ± 7·19	
13·63	0	40·1 %
27·26	0	
272·76	0	
1 % ethanol	355·25 ± 20·62	

* Not significantly different from control ($0·5 > P > 0·1$).
† Significantly different from control ($P < 0·001$)

EE = exoerythocytic

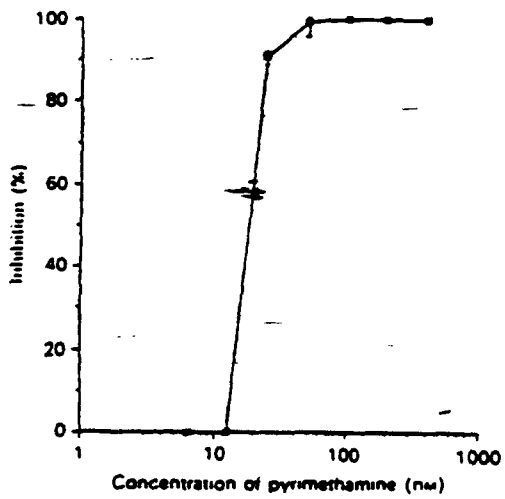


Fig. 3. Concentration-dependent effect of pyrimethamine on the numbers of *Plasmodium berghei* EE stages developing in HepG2 cells, expressed as percentage inhibition compared to control preparations receiving medium without drug. EE cultures were treated with drug 3 h after sporozoite inoculation and were examined 45 h later. Each drug concentration was tested on 4 replicates. Error bars are standard errors.

Table 17 Effect of pyrimethamine on the numbers of *Plasmodium berghei* EE stages developing in cultured HepG2 cells

Concentration of pyrimethamine (nM)	Mean no. of EE forms ±s.e. ($n = 4$)
0	145·50 ± 21·31
12·56	169·25 ± 4·66*
25·13	14·00 ± 0·71
50·26	1·25 ± 0·75
100·52	0
201·04	0
402·07	0
0·1 % ethanol	140·00 ± 5·49†

* Not significantly different from control ($0·5 > P > 0·1$).
† Not significantly different from control ($P > 0·5$).

(b) Proguanil

Activity of proguanil and cycloguanil was measured against the liver stages of *P. yoelii*, *P. cynomolgi* and *P. knowlesi*. In the study by Eriksson *et al.*, 1991 (Trans. Roy. Soc. Trop. Med. Hyg. 85: 725), Balb/c hepatocytes were inoculated with the sporozoites of *P. yoelii* and the cultures incubated for 3 hours and the medium changed. Different concentrations of proguanil and its metabolites were added and the cultures incubated for 48 hours with a change of medium containing the drug at 24 hours of incubation. The number of schizonts was determined by Giemsa staining. Proguanil did not show any activity at a concentration of 100 nM (25.4 ng/ml). The results in Figure 4 show that cycloguanil inhibited the development of schizonts at a concentration of 10 nM (2.52 ng/ml).

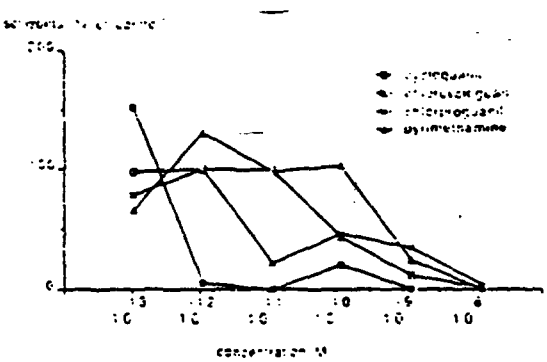


Figure 4 Effects of cycloguanil, chlorproguanil, chlorcycloguanil and pyrimethamine on the growth of liver schizonts of *Plasmodium yoelii yoelii* in the hepatocyte *in vitro* system.

In another study the activity of proguanil/cycloguanil was tested using rhesus monkey hepatocytes infected with *P. cynomolgi* or *P. knowlesi* sporozoites. The results in Table 18 show that proguanil and cycloguanil reduced the development of the schizonts at a concentration of ≥ 10 ng/ml and 1 ng/ml respectively. The authors have stated that the drugs were toxic to the hepatocytes at a higher concentration. Results in Table 18 show cycloguanil to be toxic at a concentration of 100 ng/ml. Chloroquine did not exhibit significant activity against the development of the exoerythrocytic schizonts.

TABLE 18

Results of 4 in vitro experiments showing the schizonticidal activity of 6 compounds on P. cynomolgi bastianelli, P. cynomolgi *(Cambodian strain), and* P. knowlesi *(H strain)*

Drug	Concentration µg/ml	*P. cynomolgi*		*P. knowlesi*		Cytotoxicity*
		bastianelli	Cambodian	Experiment 1	Experiment 2	
Control		68, 79	6, 8	30, 17	103, 121	
Primaquine	1	0	0	0	0	+
	10^{-1}	0	0	0	0	
	10^{-2}	6	0	0	0	
	10^{-3}	49	3	2	65	
	10^{-4}	24	7	5	117	
WR238605	1	0	0	0	0	++
	10^{-1}	0	0	0	0	
	10^{-2}	5	1	0	26	
	10^{-3}	12	5	4	55	
	10^{-4}	15	9	28	57	
	10^{-5}	74		8		
WR242511	1	0	0	0	0	++
	10^{-1}	0	0	0	0	+
	10^{-2}	0	0	1	2	
	10^{-3}	1	3	0	12	
	10^{-4}	55	7	4	41	
	10^{-5}	64		17		
Pyrimethamine	10^{-2}	0	0	0	0	
	10^{-3}	24	1	3	26	
	10^{-4}	63	3	20	141	
	10^{-5}	55	10	5	102	
Proguanil	10^{-2}	0	0	0	0	
	10^{-3}	21	4	0	10	
	10^{-4}	56	4	12	55	
	10^{-5}	62	9	15	75	
Cycloguanil	10^{-2}	0	0	0	0	+
	10^{-3}	0	0	1	3	
	10^{-4}	33	4	3	23	
	10^{-5}	79	6	12	50	
Chloroquine	1	36	6	4	82	+++

Values indicate total number of schizonts in 1 culture dish (2 hepatocyte monolayers). For control plates, 2 culture dishes were used in each experiment (4 monolayers).

* + = A few hepatocytes vacuolated at the periphery of the monolayer; ++ = all hepatocytes vacuolated or dead at the periphery of the monolayer; +++ = all hepatocytes vacuolated or dead.

It is of note that hypnozoites (the latent stages) were not reliably observed. The activity of the drugs under these experimental conditions reflects their effect after the invasion of the sporozoites into the hepatocytes.

B. Activity against the erythrocytic parasites *in vivo*

(i) Activity of atovaquone against *Plasmodium falciparum:*

The activity of atovaquone was measured in 6/8 separate experiments (# AOT-2 to AOT-7; Table 19) conducted in monkeys (*Aotus trivirgatus*) infected with the erythrocytic stages of the T9/96 clone (FCW-1/Nigeria strain) of *P. falciparum* (report no. BUAC/88/CI). This strain has been shown to be highly pathogenic leading to death within 3 – 4 days of patent infection in untreated monkeys. For most of the experiments, unless specified otherwise, monkeys were infected with 5 x 10^5 parasites by the intravenous route. All animals were dosed orally with a catheter usually on day 9 post-infection when parasitemia reached 1 – 3 %.

AOT-2: Two splenectomized monkeys were treated with either 10 or 1 mg/kg b.i.d. doses of atovaquone for 7 days. Although parasitemia was cleared in both of the infected monkeys, recrudescence occurred 18 days after the initial clearance (Table 19). The monkey treated with the 10 mg/kg b.i.d. dose cleared the parasitemia after 8 days of recrudescence. The total duration of observation was not specified. The monkey initially treated with the low dose (1 mg/kg b.i.d.) of atovaquone was then administered a higher dose (10 mg/kg b.i.d. for 5 days followed by 10 mg/kg for 2 days). The sponsor has stated that the blood smears were negative for parasite until 2 days after the last dose. Recrudescence of parasitemia for prolonged period of time was not measured (i.e., beyond 48 hours).

AOT-3: In this study a higher concentration of the inoculum was used for infection (2 x 10^7). The sponsor stated that the efficacy side of this experiment failed. Six monkeys were tested in this experiment and administered different regimens of the drug. A dose of up to 10 mg/kg for 6 days was not effective in reducing parasitemia (Table 19).

AOT-4: One monkey was administered 10 mg/kg b.i.d. for 7 days. Blood smears were negative for parasites on day 9 of treatment and no recrudescence was observed (Table 19).

AOT-5: Different concentrations of the inocula were used to infect 6 monkeys (i.e., 10^5, 5 x 10^5 or 10^6). Five of the infected monkeys were treated with 10 mg/kg atovaquone for either 5 or 7 days. Parasitemia was cleared on day 6 and no recrudescence was observed (Table 19). One monkey was left untreated. The control (initially untreated) monkey died on day 11 with parasitemia of 16% although treatment with chloroquine had been initiated. The time of initiation of treatment with chloroquine was not specified.

Table 19

	PROTOCOL						EFFICACY		PLASMA LEVELS						
Exp.No.	Protocol No.	Formulation (a)	Batch No.	Dosing Regime	Total Drug	No. Patients	Parasitaemia at treatment	Day initial clearance	Recrudescence (b)	Retreatment	Plasma Levels (c)	Maximum level obt. (d)	Mean conc. during	Plasma level day 5 (e)	REPORT
CYHD-1	-	Burtford	CE	10mg/kg	10mg	1	-	-	-	-	Bio	630	24	40	
CYHD-4	-	Burtford	CE	10mg/kg	10	3	-	-	-	-	Bio GC	170 260	12 6/12	12 -	GTAC 86-5C
			CE	20mg/kg	20	3	-	-	-	-	Bio	160	30	-	
		Formulation I	CE	10mg/kg	10	3	-	-	-	-	Bio	540	6	27	
ADT-2 (Solomon-Control)	GTAC 86-5C	Burtford	CE	10mg/kg 000x7	112	1	5%	7	Day 10	None	Bio	342	120	342	GTAC 86-5C
				1mg/kg 000x7	11.2	1	2%	5	Day 10		Bio	72	120	72	
ADT-3	GTAC 86-13C	Burtford	CE	20mg/kg	20	1	1.3%	Not cleared	-		Bio	294	42	-	
				10mg/kg x 6	60	1	2%	Not cleared	Terminated		Bio	2,088	96	2,491	GTAC 86-13C
				10mg/kg x 4	40	1	2%	Not cleared	Died		Bio	1,236	120	1,231	
				10mg/kg x 2 (48hrs apart)	20	1	1%	Not cleared	Terminated		Bio	271	72	52	
				1mg/kg x 7	7	1	2%	Not cleared	Terminated		Bio	377	48	388	
				0.1mg/kg x 7	0.7	1	2%	Not cleared	Died		Bio	388	96	157	
ADT-4	-	Burtford	CE	10mg/kg 000x7	140	1	2%	9	None	-	Bio GC	2,168 2,000	168 168	770 748	GTAC 86-5C
ADT-5	GTAC 86-20C	Burtford	CE	10mg/kg x 7	70	2	1-2.6%	6	None	-	Bio	388	48	139	GTAC 86-13C
		Formulation I	CE	10mg/kg x 7 x 5	70 50	1 2	1-2%	6	None	-	Bio	1,662	72	256	
ADT-6	GTAC 86-13C	Formulation I	IM	1mg/kg x 7	7	3	1.2-1.3%	3-4	None	-	Bio GC	44 243	144 72	27 252	GTCR 87-16C
				1mg/kg x 5	5	3	0.8-1.2%	3-4	Day 24-28	None	Bio GC	81 212	48 48	30 152	GTAC 86-19C
				1mg/kg x 3	3	2 1	1.2-1.3% 1.2%	4 *	None Day 7	- None	Bio GC	30 272	144 48	30 27	GBAR/87/014/C
ADT-7	GTAC 86-4C	Formulation I IM plus contraction		10mg/kg x 2 (48hrs apart)	20	1	2.2%	4	None	-	Bio GC	6,600 8,300	24 24	120 300	GTCR 87-5C
				30mg/kg	30 30	4 1	1.7-2.0% 3.5%	3-5 5	None 4	- 30mg/kg SAMC Day 10	Bio GC	4,000 5,000	24 24	40 60	GTCR 87-16C
				20mg/kg	20	3	1.0-2.3%	3-6	None	-	Bio GC	2,300 6,220	24 24	170 60	GTCR 87-4C
				20mg/kg x 2 (48-hrs apart)	40	2	3.1-4.2%	6-7	None	-	Bio GC	(30,13077) 8,870	24 24	140 120	GBAR/87/015/C
ADT-8	GTCR 86-3C GTCR 87/7	0.25% colloidal CF/		10mg/kg	10	2	- ND	- ND	- ND	- ND	Radio Bio GC	597 1,357 477	- 7 7	7 60 60	GBAR/87/023/C

a) See Appendix 1

b) Days post treatment.

c) Method of plasma level determination

Bio bioassay
GC gas chromatography
Radio use of radiolabelled compound

d) Maximum level measured, this will depend on when samples were taken and is not necessarily the peak. The highest level from the individual monkeys used is quoted.

e) The maximum level measured within a group is quoted.

* One animal died on 2nd day of treatment at 1.8%.

ND = not done

AOT-6: Monkeys were treated with either chloroquine (10 mg/kg/day for 7 days) or atovaquone (1 mg/kg/day for 3, 5 or 7 days). Each group consisted of 3 monkeys. Results in Table 19 show that the blood smears were negative by day 4 from all the monkeys treated with atovaquone. Recrudescence was observed between days 24 and 38 in all of the animals treated with 1 mg/kg for 5 days and another monkey treated for 3 days. Recrudescence was not observed in any of the monkeys treated for 7 days. The results of the parasitemia in monkeys treated with chloroquine are not shown. The sponsor has stated that parasitemia was not controlled and one monkey died. On day 5 of chloroquine administration the remaining 2 monkeys were treated with atovaquone (4 mg/kg followed by 3 mg/kg b.i.d. for 3 days and 3 mg/kg/day for 3 days). The sponsor stated that this lead to clearance of parasitemia (data not shown).

AOT-7: In another experiment, monkeys were treated with 1 or 2 doses of atovaquone (10 or 20 mg/kg). The results in Table 19 show initial clearance of parasitemia by day 7. Recrudescence was observed in one monkey that was administered a single dose of 10 mg/kg on day 8. It appears that the effectiveness of the drug may depend on the severity of infection. For example, monkeys with initial parasitemia of 1.5 to 2.3% were free of parasites between 3 to 6 days post-treatment with a single dose of atovaquone (20 mg/kg). Monkeys with parasitemia between 3.1 – 6.2 % continued to show an increased parasite count and were administered a second dose at 48 hours after the first dose. Although the number of animals in each group was very small these observations along with those made in experiment AOT-3 suggest that the effectiveness of atovaquone may decrease with an increase in the severity of infection.

In another study by Hudson *et al.*, 1991 [Drugs Exptl. Clin. Res. 17: 427 (possibly the same as that described above)], the activity of atovaquone was measured in *Aotus trivirgatus* infected with 5 x 10^5 *P. falciparum* parasites by the intravenous route (Wellcome FCW-1/Nigeria). Treatment with atovaquone (1 mg/kg for 3, 5, or 7 days) by the oral route was initiated on day 8 of infection i.e., at the time the parasitemia was shown to be 1 – 2%. Chloroquine (10 mg/kg) was used as a comparator. The authors stated that parasitemia was cleared in all monkeys within 3 to 7 days of treatment. Treatment with atovaquone for 7 days was reported to produce a complete cure. Recrudescence was observed in monkeys treated for a shorter duration (i.e., 3 or 5 days). Such a recrudescence was, however, followed by spontaneous clearing of the parasite. Chloroquine was reported to be ineffective in clearing parasitemia. However, no data were provided in the report for any of these experiments.

(ii) Activity of atovaquone against other *Plasmodium* species

In a study by Hudson *et al.*, 1991 (Drugs Exptl. Clin. Res. 17: 427), activity of atovaquone was measured in CD1 mice infected with *P. yoelii* by the intravenous route with 0.1 ml of 3 x 10^6 parasitized erythrocytes/ml inoculum. Treatment with 7 doses of atovaquone was initiated a few hours post-infection and was given by the oral route over a period of 3½ days. In another group of mice treatment with a single dose was initiated at different time intervals before or after infection (-24 to +24 hours). Blood smears were examined for the presence of parasites on day 5 post-infection. The results in Table 20 are expressed as 50 % effective dose (ED_{50}). It is difficult to evaluate the effectiveness of the drug in clearing parasitemia or improving survival based on calculated ED_{50} values. In addition, no other known antimalarial agent was included for comparison.

Table 20 In vivo efficacy of naphthoquinones of interest against P. yoelii with varying dosing schedules in 0.25% celacol [ED_{50} in mg/kg]*.

	566C80	59C80	306C80	58C80	296C80
7 x p.o.	0.052 [0.04–0.06]	0.098 [0.08–0.11]	0.34 [0.29–0.41]	0.78 [0.56–1.07]	> 10 mg/kg
1 x p.o. 24 h post infection	0.072 [0.03 –0.165]	0.326 [0.304–0.353]	7.8 [3.25 –12.88]	8.99 [5.81–13.9]	NT
1 x p.o. 5 h prior infection	0.11 [0.07–0.16]	1.02 [0.38–2.7]	19.7 [10.9–35.4]	NT	NT
1 x p.o. 12 h prior infection	0.3 [0.15–0.6]	0.96 [0.66–1.41]	NT	NT	NT
1 x p.o. 24 h prior infection	0.82 [0.65–1.04]	4.08 [3.21–5.19]	NT	NT	NT

* NT = not tested; [] = 95% confidence limits.

In another experiment the activity of atovaquone was measured in mice infected with either *Plasmodium yoelii* or *P. berghei* which were known to be sensitive or resistant to chloroquine, mefloquine or pyrimethamine. The results, expressed as calculated ED_{50} and ED_{99} values (Table 21), indicate that the activity of atovaquone against these specific drug sensitive and drug resistant strains is comparable.

Table 21. Comparative antimalarial activity against drug-resistant rodent strains (7 doses orally in celacol mg/kg)*

		Sensitive strain		Drug resistant strain		
566C80		ED_{50}	ED_{90}		ED_{50}	ED_{90}
P. yoelii	a)	0.027	0.089	chloroquine resistant	0.020	0.089
		0.028	0.072		0.026	0.077
	b)	0.061	0.115	pyrimethamine resistant	0.036	0.108
		0.065	0.15		0.03	0 11
P. berghei	c)	0.029	0.009	mefloquine resistant	0.030	0 110
		0.057	0.116		0.068	0 177
Chloroquine						
P. yoelii	a)	2.36	6.31	chloroquine resistant		parasites still present at 210 mg/kg
		2.18	3.11		3.59	12.73
	b)	2.20	4.16	pyrimethamine resistant	2.23	4.20
P. berghei	c)	1.95	3.50	mefloquine resistant	2.71	4.31
		1.72	3.03		3.15	(poor limits)
Pyrimethamine						
P. yoelii	a)	0.024	0.093	chloroquine resistant	0.019	0.110
	b)	0.024	0.079	pyrimethamine resistant	4.33	23.24
		0.03	0.014		5.44	55.78
Mefloquine						
P. berghei	c)	0.710	1.512	mefloquine resistant	6.19	9.65
		1.23	1.81		2.89	10.03
		1.53	2.87		3.07	10.43

* Results of individual experiments comparing normal and resistant strains in the same test.

(iii) Activity of atovaquone, proguanil, or atovaquone + proguanil against *Plasmodium yoelii:*

CD1 mice were infected with a hydroxynaphthoquinone resistant strain of *Plasmodium yoelii* (report no. BGEJ/96/0001) by the intravenous route (10^6 parasitized erythrocytes) and treated with either atovaquone or proguanil alone or a combination of atovaquone + proguanil (in different proportions). Treatment was initiated immediately after infection by the oral route. On day 5, tail vein blood smears from mice were screened for the presence of parasites. The results indicate that proguanil alone at a dose of 20 mg/kg was effective in inhibiting parasitemia by 97% (Tables 22 to 24). Atovaquone ($\leq$ 200 mg/kg) alone was not very effective in clearing parasitemia. A combination of atovaquone (100 or 200 mg/kg) with proguanil (20 mg/kg) produced complete clearance of parasitemia. A lower dose of proguanil (5 or 10 mg/kg) with atovaquone (200 mg/kg) was also effective in clearing parasitemia by $\geq$ 99%. The effect of the combination appears to be additive. No antagonistic effect of the combination was observed. Rates of relapse/recrudescence of infection after discontinuation of treatment were not measured. The sponsor has stated that the absorption and metabolism of the two drugs in mice is different from humans. Therefore the drug levels achieved in plasma in mice may not be relevant to those observed in humans.

Table 22 % parasitaemia and % inhibition of the different drug combinations

Compound 1	mg kg^{-1}	Compound 2	mg kg^{-1}	%p	%p	%p	%p	%p	Mean	% inhibition
		CONTROLS	–	98	99	98	98	99	98.4	
Atovaquone	200	Proguanil→	20.0	0	0	0	0	0	0	100
↓	200		10.0	0	0	0	0	0	0	100
	200		5.0	2	3	4	2	3	2.8	99
	200		2.5	24	20	25	28	27	24.8	75
	200		0.0	66	35	62	31	33	45.4	54.0
Atovaquone	100	Proguanil	20.0	0	0	0	0	0	0	100
	100		10.0	36	30	10	14	26	23.2	76.4
	100		5.0	54	52	49	53	48	51.2	48.0
	100		2.5	61	59	64	66	52	61.4	38.0
	100		0.0	98	97	98	97	99	97.8	0.7
Atovaquone	50	Proguanil	20.0	3	2	5	6	2	3.6	96.3
	50		10.0	50	51	49	49	53	50.4	49.0
	50		5.0	96	94	97	96	93	95.2	3.3
	50		2.5	98	98	97	99	97	97.8	0.9
	50		0.0	99	98	98	98	97	98.4	0.0
Atovaquone	25	Proguanil	20.0	2	3	6	6	3	4.0	96.0
	25		10.0	61	62	60	60	65	61.6	37.4
	25		5.0	97	98	98	96	96	97	1.5
	25		2.5	98	97	98	98	97	97.6	0.9
	25		0.0	98	95	99	98	98	98.2	0
Atovaquone	12.5	Proguanil	20.0	2	5	6	6	4	4.6	95.3
	12.5		10.0	65	64	60	66	70	65.0	34.0
	12.5		5.0	98	99	98	97	97	97.8	0.7
	12.5		2.5	99	98	98	98	98	98.2	0
	12.5		0.0	98	98	98	99	99	98.4	0
Atovaquone	0.0	Proguanil	20.0	3	2	4	2	2	2.6	97.3
	0.0		10.0	89	92	87	90	94	90.4	8.2
	0.0		5.0	95	98	96	95	97	96.2	2.3
	0.0		2.5	98	97	98	97	99	97.8	0
		CONTROLS	–	99	98	98	99	99	98.6	0

• %p = percent parasitaemia in individual animal

Table 23 Percentage inhibition of atovaquone/proguanil combinations

Atovaquone→	200 mg kg^{-1}	100 mg kg^{-1}	50 mg kg^{-1}	25 mg kg^{-1}	12.5 mg kg^{-1}	0.0 mg kg^{-1}
Proguanil↓						
20.0 mg kg^{-1}	100	100	96.3	96	95.3	97.3
10.0 mg kg^{-1}	100	76.4	49.0	37.4	34.0	8.2
5.0 mg kg^{-1}	99.0	48.0	3.3	1.5	0.7	2.3
2.5 mg kg^{-1}	75.0	38.0	0.7	0.9	0	0.0
0.0 mg kg^{-1}	54.0	0.7	0	0	0	0

Table 24 ED_{50} s of atovaquone/proguanil combinations

Compound	ED50 mg kg^{-1}	95% Confidence limits	Atovaquone (Normalised ED_{50})	Proguanil (Normalised ED_{50})
Atovaquone	196.15	200.1-192.2	1.0	0.0
+2.5 mg kg^{-1} PRG	127.13	170.2-94.9	0.65	0.19
+5.0 mg kg^{-1} PRG	101.72	104.6-98.9	0.52	0.38
+10 mg kg^{-1} PRG	51.72	93.3-31.33	0.26	0.75
+20 mg kg^{-1} PRG	4.98	26.5-0.936	0.025	1.51
Proguanil	13.25	15.03-11.7	0.0	1.0
+12.5 mg kg^{-1} ATO	11.32	11.73-10.93	0.85	0.06
+25.0 mg kg^{-1} ATO	11.02	11.29-10.75	0.83	0.13
+50 mg kg^{-1} ATO	10.10	10.23-9.93	0.76	0.25
+100 mg kg^{-1} ATO	5.08	9.96-2.59	0.38	0.51
+200 mg kg^{-1} ATO	2.08	2.66-1.63	0.16	1.02

C. Activity *in vivo* against the development of hepatocyte stages of *Plasmodium* species (causal prophylaxis):

Proguanil:

Rhesus monkeys were infected with the sporozoites of *P. cynomolgi* by the intravenous route. Treatment with proguanil (10 mg, administered intramuscularly) or pyrimethamine (1 mg by the oral route) was initiated immediately after infection for 4 to 5 days respectively. Results in Table 25 indicate that treatment with proguanil delayed the onset of parasitemia as compared to the untreated control. Microscopic examination of the day 7.5 liver biopsy showed the absence of normal schizonts in monkeys treated with proguanil or pyrimethamine. However, proguanil had no effect on the hypnozoite stage of the parasite. Retarded schizonts (slow growing forms) were also observed. Both hypnozoites and retarded schizonts can lead to relapse.

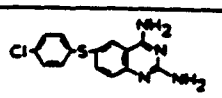
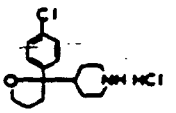
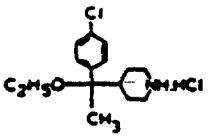
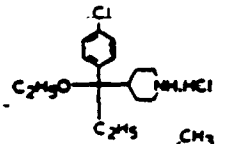
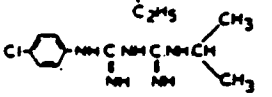
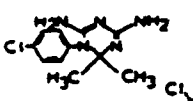
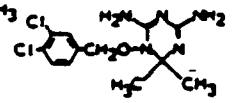
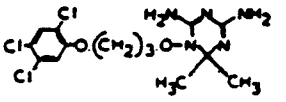
Table 25 Number of parasites seen in liver biopsies

Monkey	Day	Treatment	Sporozoite numbers	Schizont secuons/cm^2 Normal	Retarded	Hypnozoites/cm^2	Parasitaemia
1	7·5	None	2·1 ×10^6	7(3)	0(0)	2(2)	7.5
	62			0(0)	0(0)	2(1)	
2	7·5	Pyrimethamine	2·1 ×10^6	0(0)	0(1)	2(2)	20
	30			(0)	(0)	(4)	
	62			0	0	1	
3	7·5	None	9·75×10^6	40(39)	0(0)	7(7)	7.5
4	7·5	Proguanil	9·75×10^6	0(0)	18(0)	28(15)	48

Figures not in parentheses determined in the People's Republic of China; those in parentheses, in the USA.

In another study (Peters *et al.*, 1975, Am. Trop. Med. Parasitol. 69: 311), the causal prophylactic effect of proguanil and cycloguanil was examined in mice infected with the sporozoites of *P. yoelii nigeriensis* (known to be resistant to chloroquine) by the intravenous route. A single dose of cycloguanil/proguanil was administered 3 hours following inoculation of the sporozoites. Forty-eight hours following infection, erythrocytes infected with the same parasites were injected into a subgroup of animals by the subcutaneous route. The results expressed as MFAD (minimally fully active dose) show proguanil and cycloguanil to be effective at a dose of 3-10 and 1-2 mg/kg, respectively (Table 26). These results are based on examination of blood smears for the presence of parasites up to 14 days post-infection. Untreated mice developed parasitemia at a rate of 2% by 5-6 days post-sporozoite inoculation. Liver tissue was not examined for the presence of parasites.

TABLE 26

Compound name or no. and salt	Structure	MFAD mg/kg sc x 1
WR 135,403		0.1 - 0.3
BA 41,799		10 - 30
WR 179,305 fumarate		3 - 10
WR 184,520 HCl		100 inactive
proguanil HCl		3 - 10
cycloguanil HCl		1 - 2
clociguanil HCl		1 - 3
WR 99,210 HBr		1 - 3

Atovaquone:

The study by Davies *et al.*, 1989 (Acta Leidensia 58: 115) measured the prophylactic activity of atovaquone in Brown Norway rats infected with *P. berghei* by the intravenous route. Rats were treated with a single dose (0.1, 1 or 10 mg/kg) of atovaquone 3 hours post-infection. Results in Table 27 show that atovaquone at a dose of ≥ 1 mg/kg was effective in inhibiting the development of parasitemia. – The 0.01 mg/kg dose of atovaquone was effective in reducing peak parasitemia. A subgroup of rats was also infected with erythrocytic parasites by the intraperitoneal route, 48 hours post-infection with the sporozoites. Parasitemia was reduced in the subgroup of animals treated with the high dose of atovaquone (10 mg/kg) suggesting a residual inhibitory effect on the erythrocytic stages.

Table 27 Summary of the effects of 566C80 on *P. berghei* infections in sporozoite-infected rats.

Dose of 566/C80 mg/kg	Proportion of rats developing a parasitaemia	Mean pre-0.4% parasitaemia period/days ± SE[a]	Mean peak % parasitaemia ± SE
0	3/3	7.69 ± 0.47	1.66 ± 0.24
0.1	3/3	9.39 ± 1.22[b]	0.74 ± 0.18[d]
1.0	0/3	> 15[c]	0
10.0	0/3	> 15[c]	0

[a] Time from sporozoite-inoculation to the attainment of a parasitaemia of 0.4%.
[b] Not significantly different from the control (P > 0.1).
[c] No parasitaemia detected 15 days after sporozoite inoculation.
[d] Significantly different from control (0.05 > P > 0.02).
All rats were dosed with 566C80 + Celacol or with Celacol alone 3 hours after sporozoite inoculation.

The residual activity of the drug was also measured by transferring blood from infected rats at 42 hours post-sporozoite inoculation into uninfected, untreated mice by the intraperitoneal route. The results in Table 28 show that blood from rats treated or untreated and infected with both the sporozoites and the erythrocytic parasites (group X, Table 28) was infectious for naïve mice thereby indicating no residual activity of the drug. However, mice infected with the blood from rats infected with the sporozoites alone (group S, Table 28) and treated with atovaquone did not develop parasitemia up to 13 days post-infection, which provides evidence of an inhibitory effect of atovaquone on the development of liver schizonts.

Table 28 Summary of *P. berghei* infections in mice inoculated with blood from infected rats which were treated with 566C80.

Dose of 566C80/ mg/kg	Proportion of mice developing a parasitaemia		Mean pre-2% parasitaemia period/days ± SE[a]	
	S	X	S	X
0	2/3	3/3	8.00 ± 0.28	4.69 ± 0.24
0.1	0/3	3/3	> 13	4.48 ± 0.06
1.0	0/3	3/3	> 13	4.63 ± 0.17
10.0	0/3	3/3	> 13	4.63 ± 0.09

[a] Time from the inoculation of infected blood to the attainment of a parasitaemia of 2%.
S Sporozoite-infected donor rats.
X Sporozoite- and blood stage-infected donor rats.

These studies support the inhibitory effect of proguanil and atovaquone alone on the development of schizonts. However, one cannot comment on the effect of the drugs on the sporozoites i.e., migration of the infective forms to the liver, hepatic cell invasion, etc. The effect of pretreatment of mice with proguanil or atovaquone followed by inoculation of sporozoites through the bite of mosquitoes was not measured.

The prophylactic effect of atovaquone and proguanil alone was measured in human volunteers following inoculation by mosquito bite (for details see Medical Officer's review).

D. *In vivo* activity against the development of gametocytes of *Plasmodium* species within the mosquitoes:

(a) Atovaquone

In a study by Fowler *et al.*, 1994 (Parasitol. 108: 383) mice were infected with parasitized erythrocytes of *P. berghei* ANKA strain (a gametocyte producing clone) by the intraperitoneal route then treated with atovaquone on day 4 post-infection when parasitemia was observed to be in the range of 4 – 10 %. The mosquitoes were fed on infected mice after 8 hours of treatment with atovaquone (when drug levels were stated to have reached a maximal level), for approximately 15 to 20 minutes. From days 8 to 11 post-infection, mosquitoes were examined for the presence of oocysts by enzyme linked immunosorbent assay (ELISA) using antibodies specific for detecting circumsporozoite protein (CSP) in the midgut and abdominal extracts. Thorax and abdominal extracts were also used for examining sporozoites. The oocyst count was shown to be decreased in mosquitoes fed on mice pretreated with $\geq$ 0.1 mg/kg atovaquone as compared to the untreated group (Figures 5 and 6). Mosquitoes fed on mice pretreated with atovaquone ($\geq$ 1.25 mg/kg) were not capable of infecting naïve mice (Table 29).

Blood smears from mice were examined before treatment and immediately prior to mosquito feeding. No change in gametocyte count was observed.

These studies show that treatment of mice with atovaquone decreases mosquito infectivity and the potential for spread of infection to naïve uninfected animals.

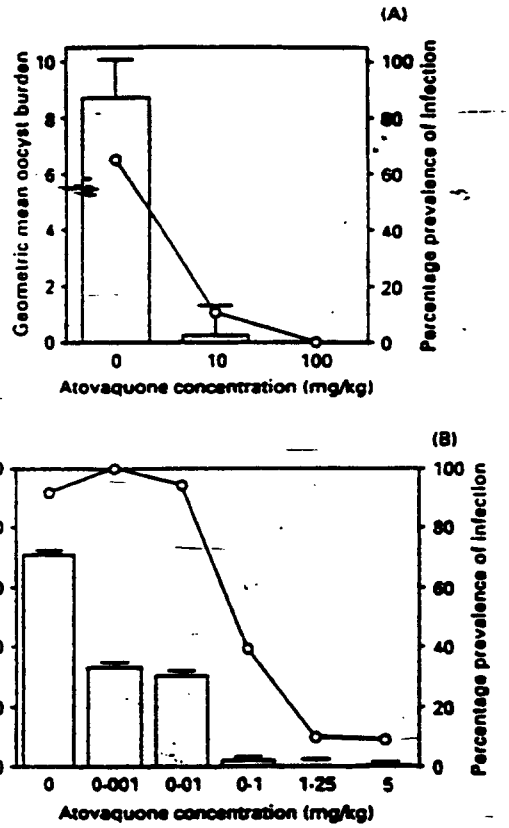


Fig. 5. The effect of atovaquone administered with the gametocyte feed on mean oocyst numbers/mosquito and the prevalence of oocyst infection. Mosquitoes were fed on mice gavaged with 0–100 mg/kg atovaquone in 0·25 % methyl cellulose, 8 h previously. Oocyst counts/mosquito (⊠) and the percentage of mosquitoes infected (–O–) are illustrated. The error bars show the standard error for the geometric means (see Materials and Methods section). (A) $n = 43–56$; (B) $n = 19–36$.

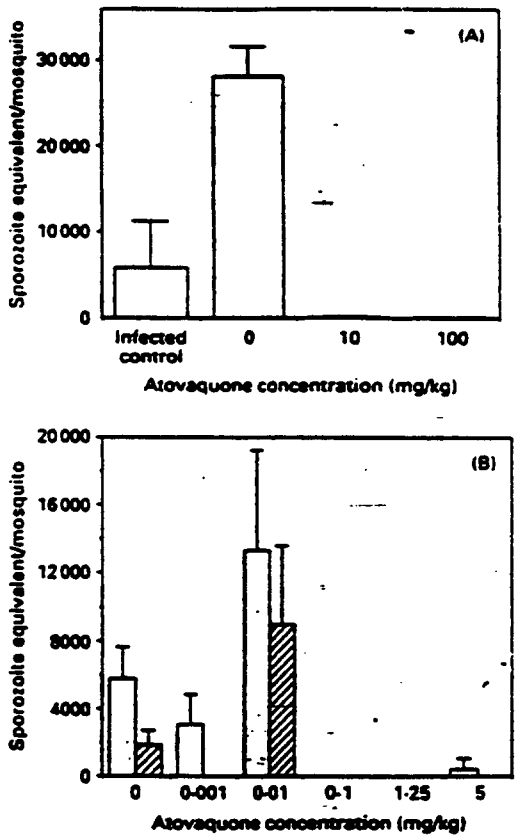


Fig. 6. The effect of atovaquone administered with the gametocyte feed on resultant sporozoite numbers. Mosquitoes were fed on mice gavaged with 0–100 mg/kg of atovaquone in 0·25 % methyl cellulose, 8 h previously. Mosquitoes were frozen on days 20–22 p.i. and sporozoites measured by CSP ELISA. Mean sporozoite numbers/mosquito thorax (⊠) and /mosquito abdomen (▨), were derived from standard distributions. The error bars show the standard error for each treatment group (A) $n = 40$; (B) $n = 15–54$.

Table 29. Effects of atovaquone on the transmission of *Plasmodium berghei* to naive, susceptible mice

(Each mosquito group was fed on 1 mouse which had been administered 0·001–100 mg/kg atovaquone or diluent (0 mg/kg) prior to the feed. On day 20 p.i., these groups of mosquitoes were allowed to feed on 2 or 3 anaesthetized naive, susceptible (Balb/c) mice. Blood smears were taken from the mice 7–14 days after the second mosquito feed, and checked for asexual parasites. All infected mice had a patent parasitaemia on day 7 after the feed. Mice which were fed on mosquitoes in the 0·1–100 mg/kg treatment groups had not developed infections by day 14 after the second mosquito feed.)

Experiment no.	Atovaquone dose (mg/kg)	No. of mosquito groups tested	No. of mice infected/no. tested
3	0	1	2/2
	5	1	0/2
	10	1	0/2
	25	1	0/2
	50	1	0/2
	100	1	0/2
4	0	1	2/3
	0·1	2	0/3
	1·25	2	0/4
	5	1	0/2
5	0	1	2/3
	0·01	2	2/4
	0·001	2	4/4
	0·1	1	N.D.*

* N.D., Not done: all mosquitoes dissected for oocyst counts.

(b) Proguanil

In a study by Omar *et al.*, 1974 (Experimental Parasitol. 36: 167) monkeys *(Macaca mulatta)* were infected with *P. cynomolgi* (by subinoculation of infected blood – route of infection not specified). Treatment with proguanil (chlorguanide, Paludrine 5.6 or 11.3 mg/kg/day) was initiated 15 days post-infection by the oral route. Mosquitoes (*Anopheles maculatus*) were fed on monkeys before initiation of treatment and at different time intervals after drug administration. The effect of pretreatment on the development from gametocytes (the stage ingested by the vector) in mosquitoes was determined by examination of the (a) gut for the presence of oocyst on day 6 to 9, and (b) salivary glands for the presence of sporozoites on days 12 to 18. Results in Table 30 indicate that peripheral blood smears were negative for the presence of parasites on day 2 or 3 of treatment with a dose of 5.6 or 11.3 mg/kg/day, respectively. Examination of the gut and salivary gland showed a reduction in the presence of oocysts (24 hours post-treatment) and an absence of sporozoites (2 hours post treatment) respectively. These findings also show that proguanil has a cidal effect on the development of sporozoites. However, the ability of such mosquitoes to infect naïve monkeys was not examined.

The activity of proguanil was compared with pyrimethamine in a separate group of monkeys treated with a single dose of pyrimethamine (3 mg/kg; equivalent to 75 mg/kg dose in humans). The results in Table 31 show an absence of sporozoites in mosquitoes (fed 15 minutes after treatment of mice) although the blood smears were positive for parasites even 4 days post treatment and development of ookinete was not altered.

TABLE 30

Effect of Chlorguanide (5.6 and 11.3 mg base/kg/day Equivalent to 150 and 300 mg in Man) on Gametocytes and Sporogony of Plasmodium cynomolgi in Macaca mulatta and Anopheles maculatus, Respectively

Time after treatment (hr)	Parasites/mm³	Gametocytes/100 WBC	Ookinetes: Formation +n	Ookinetes: Gut penetration +n	Gut dissections: Pos/Dis	Gut dissections: % pos	Gut dissections: Mean oocysts/+gut	Gland dissections: Pos/Dis	Gland dissections: % pos	Gland dissections: PGI
					X-108 (5.6 mg base/kg/Day X5)					
0	34,000	2	3	3	25/25	100	115 (1-275)	13/18	72	3.3
1	—	—	3	3	2/20	10	1.5° (1-2)	0/9	0	0
3	—	—	3	3	5/27	18	1.0°	0/8	0	0
1d 24	600	0	2	1	5/30	17	1.8° (1-3)	0/10	0	0
2d → 48	0	0	0	0	2/20	10	1.0°	0/8	0	0
3d 72	0	0	0	0	0/20	0	0	0/10	0	0
4d 96	0	0	0	0	2/20	10	3.0° (1-5)	0/7	0	0
5d 120	0	0	0	0	2/20	10	1.0°	0/10	0	0
					X-108 (11.3 mg base/kg/Day X5)					
0	120,000	75	3	3	25/25	100	438 (88-970)	20/20	100	3.8
1	—	—	3	3	12/30	40	1.58 (1-4)	1/19	5	3.0
2	—	—	3	3	12/32	38	2.4° (1-5)	0/42	0	0
3	—	—	3	3	13/30	43	1.7° (1-6)	0/40	0	0
5	—	—	3	3	11/30	37	1.8° (1-4)	0/20	0	0
8	—	—	3	3	12/34	35	1.2° (1-2)	0/20	0	0
9	—	—	3	3	7/34	20	1.5° (1-3)	0/10	0	0
24	48,400	48	3	1	1/60	2	1.0°	0/17	0	0
2d 48	750	4	1	0	2/50	4	1.5° (1-2)	0/12	0	0
3 72	0	0	0	0	3/51	6	1.0°	0/10	0	0
4 96	0	0	0	0	1/47	2	2.0	0/13	0	0
5 120	0	0	0	0	3/43	7	1.0	0/18	0	0

* 0 Some normal oocysts in addition to retarded and degenerate oocysts; ° retarded or degenerate oocysts; ( ) figures in parentheses indicate ranges; + number of plus signs indicates relative intensity of parasites observed in 6 mosquitoes (1-9: 1+, 10-99: 2+, and more than 99: 3+); PGI Positive gland index.

TABLE 31

Effects of a Single Dose of Pyrimethamine (3 mg base/kg Equivalent to 75 mg base in Man) upon Gametocytes and Sporogony of Plasmodium cynomolgi in Macaca mulatta and Anopheles maculatus, Respectively

Time after treatment (hr)	Parasites/mm³	Gametocytes/100 WBC	Ookinetes: Formation +n	Ookinetes: Gut penetration +n	Gut dissections: Pos/Dis	Gut dissections: % pos	Gut dissections: Mean oocysts/+ gut	Gland dissections: Pos/Dis	Gland dissections: % pos	Gland dissections: PGI
					X-168 (3 mg base/kg)					
0	11,900	80	3	3	30/30	100	245 (4-570)	11/12	92	3.9
½	—	—	3	3	7/32	22	2.1° (1-4)	0/8	0	0
1	—	—	3	3	9/22	41	1.6° (1-4)	0/6	0	0
1½	—	—	3	3	12/31	39	2.8° (1-6?)	0/16	0	0
2	—	—	3	3	10/27	37	1.8° (1-4)	0/8	0	0
4	—	—	3	3	10/30	33	1.1° (1-2)	0/8	0	0
8	—	—	3	2	1/20	5	1.0°	0/5	0	0
24	9,300	30	2	1	4/60	7	1.8° (1-3)	0/12	0	0
48	800	30	2	1	0/30	0	0	0/8	0	0
72	200	6	2	0	2/45	4	1.5° (1-2)	0/15	0	0
96	90	2	1	0	1/30	3	1.0°	0/18	0	0
120	130	0	0	0	3/33	9	2.3° (1-3)	0/18	0	0
					X-96° (3 mg base/kg)					
0	970	0	2	2	25/30	83	8.8 (1-22)	11/15	73	2.7
½	—	—	2	2	4/22	18	1.0°	0/8	0	0
1	—	—	2	2	3/20	7	1.3° (1-2)	0/10	0	0
1½	—	—	2	2	2/28	7	1.5° (1-2)	0/9	0	0
4			2	2	2/19	11	4.5° (1-8)	0/11	0	0
12		—	2	2	1/28	4	1.0°	0/12	0	0
2		—	2	2	4/20	20	2.0° (1-3)	0/7	0	0
24	70	0	1	0	4/21	19	1.0°	0/8	0	0
48	10	0	0	0	1/19	6	1.0°	0/5	0	0
72	0	0	0	0	0/20	0	0	0/9	0	0
96	10	0	0	0	0/20	0	0	0/7	0	0

Mechanism of Action:

The antimalarial activity of Malarone is due to its individual constituents i.e., atovaquone and proguanil.

(a) Atovaquone:

The mechanism by which atovaquone exhibits activity against *Plasmodium* species was reviewed earlier (see NDA # 20-259 microbiology review dated 10/30/92). No additional studies have been provided in this submission. The studies show that a lower concentration of atovaquone is required for inhibiting the mitochondrial proteins of *Plasmodium* (*P. falciparum* and *P. yoelii*) compared to rat liver. These results are based on the use of the same concentration of mitochondrial proteins from either rat liver or *Plasmodium*. The purity of mitochondrial proteins was not tested; therefore, it is possible that the higher activity of atovaquone against *Plasmodium* could be due to the presence of differential amounts of enzyme proteins. A comparison of the activity of the cis-form and trans-form indicates greater activity mediated by the trans-form of atovaquone in inhibiting the respiratory activity of *Plasmodium*. The site of action appears to be the bc_1 complex.

A study by Ittarat *et al.*, 1995 (Antimicrob. Agents Chemother. 39: 325) shows that atovaquone inhibits the activity of dihydroorotate dehydrogenase (DHOD), derived from *P. falciparum* crude homogenate at a concentration of 1 nM. Similar observations were made in a study by Seymour *et al.*, 1994 (Biochemistry 33: 5268) wherein atovaquone was shown to be a moderate inhibitor of dihydroorotate enzyme. The effect of atovaquone was measured by incorporation of ^{14}C bicarbonate into pyrimidine nucleotide synthesis. Results show that pyrimidine nucleotides were maximal during the development of trophozoite to the erythrocytic schizont stage. Although these studies indicate that atovaquone inhibits the activity of DHOD, a key enzyme for conversion of dihydroorotate to orotate which in turn is important for *de novo* pyrimidine biosynthesis and is also linked to the mitochondrial electron transport chain (bc_1 mitochondrial enzymes), the possibility of another drug target cannot be ruled out.

Sensitivity of DHOD to atovaquone was higher than the antiproliferative effect on the growth of the parasite. In a study by Ittarat *et al.*, 1994 (Experimental Parasitol. 79: 50), the FCR-3 strain of *P. falciparum* was cultured in the presence of atovaquone. Drug activity was measured by incorporation of ^{3}H hypoxanthine at the time of initiation of culture and incubated for 6 hours. At a concentration of 5 nM, atovaquone was shown to inhibit the incorporation of hypoxanthine by 30% whereas the activity of dihydroorotate dehydrogenase was inhibited by 90%.

(b) Proguanil:

Proguanil is metabolized to its major active metabolite cycloguanil in the presence of hepatic cytochrome p450. The review papers submitted by the sponsor show that cycloguanil inhibits the activity of dihydrofolate reductase (DHFR), an enzyme essential for the synthesis of tetrahydro folic acid. Like other protozoans, *Plasmodia* cannot utilize preformed folates but do synthesize tetrafolates *de novo* for pyrimidine synthesis. Therefore selective inhibition of tetrahydrofolic acid will inhibit the proliferation of the *Plasmodium* parasite.

The affinity of cycloguanil for the *P. berghei* enzyme is much higher than the rodent erythrocyte enzyme with K_i of 0.78 nM and 200 uM respectively. The study by Ferone, 1970 (J. Biol. Chemistry. 245: 850) was conducted using two strains of *P. berghei* (pyrimethamine-sensitive and –resistant) isolated from the infected erythrocytes by lysing, centrifugation and chromatography. The activity of cycloguanil and pyrimethamine against the 2 strains of the parasites was measured by a standard procedure. The cycloguanil K_i value for the pyrimethamine-sensitive strain was 0.78 nM (Table 32). Results also show the Ki value for the pyrimethamine-resistant strain to be about 17-fold higher than the sensitive strain. Such an increase in Ki values is attributed to an increase in enzyme content and a decrease in binding affinity, which could be due to genetic mutations.

TABLE 32

Comparison of effects of several antifolates in vivo and on H_2-folate reductases from Pb/WLTM and Pb/WLTM/60-63

Compound[a]	Pb/WLTM			Pb/WLTM/30-63			Pb/WLTM/30-63 to Pb/WLTM	
	K_i	Type of inhibition[b]	ED_{50}[c] in vivo	K_i	Type of inhibition	ED_{50} in vivo	Ratio of K_is	Ratio of ED_{50}s
	nM			nM				
Pyrimethamine								
H_2-folate not incubated[d]	0.37	NC	0.15	14	NC	>100[e]	42	>666
H_2-folate incubated[d]	0.27	C						
Cycloguanil	0.78	C	0.85	13	NC	>20	16.6	>23.5
Trimethoprim	7.1	C	75	68	NC	>2400[e]	9.6	>32

[a] Pyrimethamine, see Footnote 1; cycloguanil, 4,6-diamino-1-(p-chlorophenyl)-1,2-dihydro-2,2-dimethyl-s-triasine; trimethoprim 2,4-diamino-5-(3',4',5'-trimethoxybensyl)pyrimidine.
[b] NC, non competitive; C, competitive.
[c] Dose of drug, in milligrams per kg, which reduced the parasitemia of the treated mice to 50% of that of the untreated mice. See "Methods" for details.
[d] Enzyme and drug incubated 5 min at 37° in usual manner, with or without H_2-folate as indicated.
[e] Maximum tolerated dose.

Studies also show the mode of action of cycloguanil to be distinct from proguanil (Fidock *et al.*, 1998, Mol. Pharmacol. 54 (6): 1140). Cycloguanil was shown to act specifically on *P. falciparum* dihydrofolate reductase by *in vitro* complementation assays using *P. falciparum* parasites transfected with human DHFR. No other significant target was identified. The authors have stated

that the target for proguanil appears to be separate from cycloguanil. Also, the fact that proguanil was shown to be effective in the prophylaxis/treatment of malaria in individuals exhibiting poor metabolism of proguanil supports the antiplasmodial activity of the drug (Yeo *et al.*, 1997, Acta Tropica 67: 207; Edstein *et al.*, 1996, Trans. R. Soc. Trop. Med. Hyg. 90: 418). It is of note that DHFR inhibitors can interact with different residues surrounding the active site contributing to multiple hits on the target enzyme.

Effect on oxygen consumption:

In studies by Murphy and Lang-Unnasch, 1999 (Antimicrobial. Agents Chemother. 43: 651) the FCR3F86 strain of *P. falciparum* (erythrocytic forms) was cultured in the presence of atovaquone or proguanil and oxygen consumption measured by polarographic assays. Consistent with the activity of atovaquone as an inhibitor of cytochrome bc1 complex, results of this study showed that atovaquone inhibits up to 73% of the oxygen consumption by *P. falciparum*. Proguanil however, inhibited oxygen consumption by about 9 %. The effect of cycloguanil on oxygen consumption was not measured.

Resistance:

Resistance development by *Plasmodium* to atovaquone alone is well known. Studies reported in the literature show that *P. falciparum* can develop resistance to atovaquone *in vitro*. Atovaquone treated parasites may maintain some electron transport through an alternative branch of the respiratory chain which may favor the selection of atovaquone resistant mutants (Murphy and Lang-Unnasch, 1999, Antimicrobial. Agents Chemother. 43: 651). The site at which resistance develops is probably the same as the target site for activity (i.e., mitochondrial complex III) because the addition of orotate reversed the inhibitory effect of the drug in *in vitro* (for details see page 31, para 3, Report # BTCB-92-001C). Studies also show that treatment of malaria patients with atovaquone alone may lead to an initial clinical and parasitological cure followed by recrudescence. For example, a study by Looareesuwan *et al.*, 1996 (Am. J. Trop. Med. Hyg. 54: 62-66) shows that about one-third of the patients (from Thailand who participated in the study) with falciparum malaria and treated with atovaquone failed therapy despite an initial clinical and parasitological response. Paired isolates from these subjects demonstrated > 1000-fold increase in 50% inhibitory concentration (IC_{50}) values after therapy (from 3.3 ng/ml to 4947 ng/ml).

Resistance development to proguanil alone is also well documented (reviewed by Peters, 1990, Pharmac. Therap. 47: 499). Even a single drug exposure to a large dose of the drug may induce resistance *in vitro* and *in vivo*.

The sponsor has stated that Malarone is less likely to induce resistance because it is a combination of 2 drugs with 2 different mechanism of actions. However, the potential for development of resistance against Malarone was not investigated. Studies by Gassis and Rathod, 1996 (Antimicrob. Agents and Chemother 40: 914) show that development of resistance to two drugs with 2 separate mechanism of actions may be independent and the frequency of dual resistance may likely be a product of the frequencies of resistance to each compound. For example, erythrocytic parasites of *P. falciparum* clone W2 (from Indochina) in long term culture were shown to develop resistance in the presence of 10 nM atovaquone or 100 nM 5-fluoroorotate (an inhibitor of thymidylate synthetase responsible for pyrimidine synthesis). Fluoroorotate resistant parasites did not show cross resistance to atovaquone. However, atovaquone resistant parasites (about 30 fold resistance developed within 3 weeks) were about 5-fold more resistant to fluoroorotate. It was shown that mutants appeared at a lower frequency at higher concentrations of the drug. The number of parasites present in the culture influenced the frequency of mutants. No mutants were observed in the presence of a combination of atovaquone + fluoroorotate using one (reportedly optimal) concentration of parasites. These observations indicate that combination therapy may reduce or slow the emergence of resistance. However, whether different drug concentrations and parasitic load will alter the activity of the drug combination is not known.

In a study published by Looareesuwan *et al.*, 1996 (Am. J. Trop. Med. Hyg. 54 (1):62) about a 3-fold decrease in susceptibility was observed after treatment of 3 patients with atovaquone + proguanil for a period of 3 to 5 days (Table 33). It appears that these IC_{50} values represent atovaquone activity. Although the decrease in mean IC_{50} value is minimal this study does show that the potential for development of resistance against Malarone cannot be ruled out especially after prolonged therapy. It would be worthwhile to investigate the potential for development of resistance to atovaquone and proguanil/cycloguanil by genotypic and phenotypic analysis of isolates obtained from patients with falciparum malaria before initiation of treatment and at different time intervals after therapy with Malarone in patients who are not cured or who recrudesce.

Table 33
Effect of a combination of atovaquone + proguanil on *in vitro* susceptibility of clinical isolates

Group (n=3)	IC_{50} (ng/ml)
Pretreatment	1.2
Post-treatment	3.8

Cross-resistance:

Clones of *P. falciparum* known to be resistant to other antimalarial agents were shown to be susceptible to atovaquone and/or proguanil/cycloguanil *in vitro*. For example, in a study by Hudson *et al.*, 1991 (Drugs Exptl. Clin. Res. 17: 427), 4 laboratory strains of *P. falciparum* (T9/96, FCW-1, H-1 and D6) with high IC_{50} values to chloroquine ($\geq$ 74 nM), quinine ($\geq$ 217 nM), pyrimethamine ($\geq$ 79.7 nM), and mefloquine ($\geq$ 35 nM) showed atovaquone IC_{50} values of $\leq$ 4.3 nM.

In another study by Rathod *et al.*, 1997 (Proc. Natl. Acad. Sci. USA 94:9389) the following clones of *P. falciparum* were stated to be susceptible to atovaquone (IC_{50} of 2 nM):

- W2 [from Indochina, resistant to chloroquine (IC_{50} > 100 nM) + quinine (IC_{50} > 50 nM) + pyrimethamine (IC_{50} > 25 nM) + cycloguanil + sulfadoxine (IC_{50} > 10000 nM)],
- FCR3 (from Gambia, resistant to chloroquine + cycloguanil),
- HB3 (from Honduras, resistant to pyrimethamine),
- 3D7 (from Netherlands, resistant to sulfadoxine), and
- D6 [from Sierra, susceptible to chloroquine (IC_{50} < 15 nM) + quinine (IC_{50} < 15 nM) + pyrimethamine (IC_{50} < 1 nM) + cycloguanil + sulfadoxine (IC_{50} < 100 nM)].

In a study by Looareesuwan *et al.*, 1996 (Am. J. Trop. Med. Hyg. 54: 62), 12 isolates from patients in Thailand were shown to be susceptible to atovaquone with mean IC_{50} values of 3.3 ng/ml (i.e., 8.99 nM). These isolates were stated to be resistant to chloroquine (data not shown).

In a study by Basco *et al.*, 1995 (Am. J. Trop. Med. Hyg. 53: 388), the atovaquone IC_{50} values for chloroquine resistant and chloroquine susceptible strains were similar (Table 9, page 11). Also there was no correlation observed between atovaquone and other antimalarials IC_{50} values thereby indicating lack of cross-resistance with other antimalarials *in vitro* (Table 10, page 11). Due to the small number of isolates (n=35) tested and in the absence of correlation with clinical outcome these observations should be interpreted with caution.

In the study by Ringwald *et al.*, 1996 (Am. J. Trop. Med. Hyg. 55: 254), the IC_{50} values for cycloguanil were over 100-fold lower for the chloroquine sensitive strains as compared to chloroquine resistant strains (Table 11, page 12) thereby indicating the possibility of cross-resistance with chloroquine.

The cycloguanil/proguanil K_i values for a pyrimethamine-resistant strain were shown to be 17-fold higher than the sensitive strains of *P. berghei* (Table 32, page 32; Ferone, 1970, J. Biol. Chemistry. 245: 850) thereby indicating cross-resistance between cycloguanil and pyrimethamine. Also, the IC_{50} values of pyrimethamine-resistant isolates were 9-fold higher than the pyrimethamine-sensitive isolates (Khan *et al.*, 1987, Trans. Roy. Soc. Trop. Med. Hyg. 33: 325).

It is of note that some of the multi-drug resistant clones W2, FCR3 and 3D7 were shown to develop resistance to atovaquone *in vitro*, whereas clones D6 and HB3 did not (Rathod *et al.*, 1997, Proc. Natl. Acad. Sci. USA 94: 9389). The ability of Malarone (i.e., a combination of atovaquone + proguanil) to induce cross-resistance to other antimalarials was not investigated.

The Indications:

The sponsor has requested approval of Malarone (1) "for the **prophylaxis** of *Plasmodium falciparum* malaria infections, and (2) "for the **treatment** of acute, uncomplicated *P. falciparum* malaria.

The sponsor has stated that the indications are based on the results of the clinical trial results summarized in Table 1, line 115 of the proposed package insert. Please see the Medical Officer's review for details of the clinical trial results showing the effectiveness of Malarone in the treatment of drug resistant malaria, including malaria caused by mefloquine-resistant, amodiaquine-resistant, chloroquine-resistant, and chloroquine plus pyrimethamine/sulfadoxine-resistant parasites. The claim that Malarone is effective against resistant falciparum malaria is based on the resistance of *P. falciparum* to mefloquine and existence of cross-resistance between mefloquine and halofantrine resistant strains of the parasite.

In vitro and *in vivo* studies suggest cross resistance between mefloquine and halofantrine (Halfan label; Kertrangsee *et al.*, 1992, Southeast Asian J. Trop. Med. Public Health 23: 55). A study by Gay *et al.*, 1997 (Am. J. Trop. Med. Hyg. 56: 315) examined the correlation between the *in vitro* susceptibility of *P. falciparum* to atovaquone and to other antimalarial agents. No correlation was observed between the *in vitro* susceptibility of Asian and African strains to atovaquone and halofantrine (Table 34). However, the correlation coefficients for the *in vitro* susceptibility of African and Asian strains to drugs other than halofantrine, showed a different pattern. Among the African strains no correlation was observed between *in vitro* susceptibility to any of the drugs tested which include chloroquine, mefloquine, and halofantrine. Susceptibility of Asian strains to atovaquone was highly correlated with chloroquine and mefloquine sensitivity but not with halofantrine. These observations suggest that halofantrine resistant African and Asian isolates may be sensitive to atovaquone. However, these observations should be interpreted with caution because of the small number of observations, variations among different strains and absence of information related to clinical outcome.

TABLE 34

Correlation of atovaquone with other antimalarials among African and Asian *Plasmodium falciparum* strains*

Africa	n	r	P
AQ × CQ	62	0.14	0.27
AQ × QN	61	−0.03	0.80
AQ × MQ	62	−0.05	0.68
AQ × HF	62	−0.16	0.22
AQ × AI	62	0.02	0.85
AQ × AM	62	0.03	0.83
AQ × AE	62	−0.04	0.76
AQ × AU	62	0.06	0.65
Asia	**n**	**r**	**P**
AQ × CQ	31	0.38	0.037
AQ × QN	30	0.52	0.005
AQ × MQ	30	0.44	0.018
AQ × HF	31	0.25	0.18
AQ × AI	34	0.44	0.012
AQ × AM	34	0.50	0.004
AQ × AE	28	0.48	0.012
AQ × AU	34	0.51	0.004

* For definitions of abbreviations, see Table 1.

Effect on the immune function:

Cycloguanil has been shown to decrease lymphoproliferation of human peripheral blood mononuclear cells upon stimulation with nonspecific mitogens or antigens *in vitro* and *ex vivo*. The effect of atovaquone on lymphoproliferation or any other immunological parameters has not been reported.

SPONSOR'S PROPOSED LABEL

In a global assessment meeting held on 5/6/99, Ms. Mary Dempsey the Project Manager stated that the sponsor intends to revise the microbiology section of the label and will submit it to the Agency. The microbiology section of the label submitted with original NDA is as follows:

CLINICAL PHARMACOLGY:

Mechanism of action: The constituents of MALARONE, atovaquone and proguanil hydrochloride, interfere with two different pathways involved in the biosynthesis of pyrimidines required for nucleic acid replication. Atovaquone is a selective inhibitor of parasite mitochondrial electron transport. Proguanil hydrochloride primarily exerts its effect by means of the metabolite cycloguanil, a dihydrofolate reductase inhibitor. Inhibition of dihydrofolate reductase in the malaria parasite disrupts deoxythymidylate synthesis.

[redacted]

CONCLUSIONS:

The sponsor has requested approval of Malarone for the treatment and prophylaxis of *P. falciparum* malaria. Nonclinical studies that demonstrate the antiplasmodial activity of Malarone, a combination of atovaquone + proguanil, include the following:

Atovaquone exhibits antiplasmodial activity by inhibition of the mitochondrial respiratory chain. The site of action appears to be the bc_1 complex. Proguanil is metabolized by cytochrome P450 enzymes to cycloguanil and pCBG. The antiplasmodial activity is mainly due to its major metabolite, cycloguanil. Cycloguanil inhibits the activity of DHFR, an enzyme essential for the synthesis of tetrahydrofolates. While proguanil and pCBG also exhibit weak antimalarial activity the underlying mechanism of action is not known.

Atovaquone and cycloguanil have been shown to exhibit *in vitro* activity against erythrocytic stages of *P. falciparum* which includes laboratory strains (n=9 and n=10 respectively) and several clinical isolates from different geographic areas (Table 35). The anti-parasitic activity was measured by incorporation of ^{3}H-hypoxanthine. A majority of the studies were done by incubating the parasite with the drug for 42 hours. Results were expressed as IC_{50} values. Based on the information available either from the NDA or the literature, the atovaquone IC_{50} values varied from 0.04 to 9.6/55.7 nM (i.e., 0.015 to 3.5/20.4 ng/ml). The IC_{50} values for cycloguanil, proguanil, and pCBG varied from 17.6 to 5108 (i.e., 4.4 to 1287.2 ng/ml), 2422 to 19243 (i.e., 615.2 to 4887.7 ng/ml) and 11376 to 40662 (i.e., 2411.7 to 8620.3 ng/ml) nM, respectively, in normal medium. In medium free of *p*-aminobenzoic and folic acid the cycloguanil and proguanil IC_{50} values were 0.3 to 1360 nM (0.08 to 342.7 ng/ml) and 3239 to 13233 nM (822.7 to 3361.2 ng/ml) respectively. In the absence of raw data the precise means could not be calculated. Other DHFR inhibitors (such as pyrimethamine) and dihydropteroate synthetase inhibitors (such as sulfadoxine) have shown decreased inhibitory activity in medium containing folates and p-aminobenzoic acid (Chulay *et al.*, 1984, Am. J. Trop. Med. Hyg. 33: 325).

It is also of note that the atovaquone IC_{99} values measured against the FCW-1/Nigeria strain of *P. falciparum* were in the range of 90.9 to 2160 nM (i.e., 33 to 793 ng/ml) which are 61 to 969 times higher than the IC_{50} values obtained with different batches of the drug.

Table 35

Reference	Geographic area	Number
Atovaquone:		
Looareesuwan *et al.*, 1996, Am. J. Trop. Med. Hyg. 54: 62	Thailand	12
Gay *et al.*, 1997, Am. J. Trop. Med. Hyg. 56: 315	Central/West Africa	108
	Asia	34
Basco *et al.*, 1995, Am. J. Trop. Med. Hyg. 53: 388	Travelers (returning from Africa to France)	61
Zalis *et al.*, 1998, Am. J. Trop. Med. Hyg. 58: 630	Brazil	26
MALB2001 (GlaxoWellcome study, NDA)	Kenya	8
	Total	203
Cycloguanil:		
Ringwald *et al.*, 1996, Am. J. Trop. Med. Hyg. 55: 254	Cameroon	86
Nzila-Mounda *et al.*, 1998, Antimicrob. Agents Chemother. 42: 164	Kenya	69
Watkins *et al.*, 1987, Am. J. Trop. Med. Hyg. 37: 445	Kenya	26
Khan *et al.*, 1997, Trans. Roy. Soc. Trop. Med. Hyg. 91: 456	Kenya	21
MALB2001 (GlaxoWellcome study, NDA)	Kenya	8
	Total	210
Proguanil:		
MALB2001 (GlaxoWellcome study, NDA)	Kenya	8

The effect of atovaquone, cycloguanil and proguanil on parasitic reinvasion of erythrocytes was observed by microscopic examination and the minimum inhibitory concentrations determined. The mean $\geq$ 95% MIC values by microscopic examination against 1 to 2 laboratory strains for atovaquone, cycloguanil and proguanil were in the range of 15-20, 20 and 7595-8681 ng/ml respectively.

A combination of atovaquone + proguanil was shown to exhibit synergistic *in vitro* activity against 3 strains of *P. falciparum*. The activity of the combination of atovaquone + cycloguanil varied from synergistic to weakly antagonistic against 5 different clones of *P. falciparum*. The underlying mechanism (biochemical/molecular) for such a variability in the drug interaction pattern is not known.

The *in vitro* activity of atovaquone or cycloguanil/proguanil (added 3 hours after sporozoite invasion) was measured against the hepatic stages of *Plasmodium* species (*P. berhei*, *P. yoelii*, *P. cynomolgi* or *P. knowlesi*). The infected hepatocytes were then incubated in the presence or absence of drug for 48 hours and the number of schizonts counted by microscopic examination. The atovaquone IC_{50} value against *P. berghei* strain was 1.85 nM (0.68 ng/ml). The IC_{50} value for cycloguanil against *P. yoelii* was not calculated but appears to be in the range of 1 – 5 nM (0.25 to 1.26 ng/ml). Atovaquone/cycloguanil IC_{50} values against *P. cynomolgi* or *P. knowlesi* were also not determined. Proguanil up to a concentration of 100 nM (25.4 ng/ml) did not exhibit any activity against the development of the schizont stage of *P. yoelii*. However proguanil ($\geq$ 10 ng/ml) was effective in inhibiting the development of liver schizonts of *P. cynomolgi* and *P. knowlesi*.

The activity of atovaquone and proguanil against the life cycle stages of the parasite developing from the gametocyte stage was measured using the *P. berghei* mice and the *P. cynomolgi* monkey model respectively. In mice infected with erythrocytic forms of *P. berghei*, treatment with atovaquone decreased the infectivity of mosquitoes (fed on animals) and reduced the spread of infection to naïve uninfected animals. Similarly, in monkeys infected with erythrocytic forms of *P. cynomolgi* and treated with proguanil, the development of sporozoites in the mosquitoes was inhibited. However, the ability of such mosquitoes to infect naïve monkeys was not examined. These studies suggest that atovaquone and proguanil inhibit or reduce the transmission of Plasmodial infection by a decrease and/or inhibition of sporozoite formation.

The prophylactic effect of proguanil was examined in one rhesus monkey by infecting with the sporozoites of *P. cynomolgi* by the intravenous route. Proguanil at 10 mg/kg for 4 days was effective in delaying the onset of parasitemia. Liver biopsy on day 7.5 (i.e., 2.5 days after the last dose of the drug) showed the presence of hypnozoites and retarded schizonts. In another study conducted in mice infected with the chloroquine-resistant strain *P. yoelii nigeriensis*, proguanil was shown to be effective in preventing the onset of parasitemia. Liver tissues were not examined for the presence of residual parasites. Atovaquone was also shown to be effective in inhibiting the development of parasitemia upon infection with the sporozoite stage in rats infected with *P. berghei*. Here again, the presence of residual parasites in the liver was not examined. The prophylactic activity of atovaquone and proguanil alone has also been demonstrated in human volunteers (for details see Medical Officer's review).

Atovaquone was shown to exhibit anti-plasmodial activity in monkeys infected with 5×10^5 erythrocytic stages of *P. falciparum*. A dose of 10 mg/kg for 7 days appears to be effective in clearing parasitemia in normal monkeys. Recrudescence was observed from days 7 to 38 after discontinuation of treatment. In some monkeys parasitemia was shown to be cleared which could be due to the development of protective immune responses. Some of the animals with

recrudescence were treated by administration of a repeat dose. In 6 monkeys infected with a 40-fold higher concentration of the parasite (2×10^7) atovaquone was not effective in clearing parasitemia. Overall these studies show that 7 days of dosing with atovaquone is effective in clearing parasitemia in immunocompetent monkeys with moderate parasitemia. The effect of treatment in splenectomized monkeys is unclear.

In mice with *P. yoelii* or *P. berghei* infection (with the erythrocytic stage), treatment with atovaquone was initiated a few hours post infection and continued for 3 and one half days. The results show that atovaquone ED_{50} values are similar against strains of *P. yoelii* or *P. berghei* known to be sensitive or resistant to various antimalarial drugs (chloroquine, pyrimethamine or mefloquine). Recrudescence/relapse of infection after discontinuation of therapy was not measured.

The activity of atovaquone + proguanil was tested in mice infected with parasitized erythrocytes of a hydoxynaphthoquinone resistant strain of *P. yoelii*. Results show that proguanil (20 mg/kg) but not atovaquone ($\leq$ 200 mg/kg) alone were effective in clearing parasitemia. A combination of lower dose of proguanil (5 or 10 mg/kg) with atovaquone (200 mg/kg) was effective in clearing parasitemia. The effect of the combination appears to be additive. The recrudescence/relapse of infection after discontinuation of therapy was not measured.

Development of resistance by the *Plasmodium* parasite to atovaquone or proguanil alone is well known. The sponsor has stated that the use of 2 drugs with different mechanisms of action is less likely to induce resistance. However, the potential for development of resistance against the combination of atovaquone + proguanil was not investigated. Also, the ability of atovaquone + proguanil to induce cross-resistance against other anti-malarial agents was not investigated. The susceptibility of *P. falciparum* isolates to atovaquone was not altered by chloroquine-resistance. However, cycloguanil IC_{50} values were 117 fold higher for chloroquine-resistant strains compared to chloroquine susceptible strains. In another study, 23/28 and 9/20 chloroquine-susceptible and chloroquine-resistant isolates, respectively, were stated to be susceptible to cycloguanil (IC_{50} < 50 nM). Pyrimethamine resistant isolates were shown to exhibit higher IC_{50} (*in vitro* susceptibility) and K_i (inhibition of enzyme activity) values against cycloguanil.

Studies show the possibility of mixed infection in malaria endemic areas. For example, the analysis of DHFR phenotype by polymerase chain reaction showed that 37 isolates had different DHFR alleles and therefore were stated to be mixed isolates. Also, several alleles of merozoite surface antigen (MSA)-1 and MSA-2 were present among Brazilian isolates. The *in vitro* susceptibility of clinical isolates collected from subjects in malaria endemic areas (summarized in Table 35, page 39) suggest that the drug may be active against infections with mixed isolates.

Proposed changes in the microbiology section of the label:

1. The sponsor has described the mechanism of action under the heading and the activity of the drug *in vitro* under the heading ____. The mechanism of action and activity of the drug *in vitro* should be described as 2 separate subheadings in the ____ section of the label.

2. It should be noted that other labels for antimalarial drugs do not state the IC_{50} values in the microbiology section. However, these labels do specify the stages of the parasite against which the drug exhibits activity. Therefore, it should be specified that atovaquone and cycloguanil exhibit *in vitro* activity against the erythrocytic and exo-erythrocytic forms of *Plasmodium falciparum*. Proguanil also exhibits weak antiplasmodial activity against the erythrocytic and exoerythrocytic forms. If, however IC_{50} values are stated in the label then the values should be based on additional information available in the literature which includes testing against clinical isolates from different geographic areas and in different laboratories and not just on repeated testing of a few isolates as originally proposed by the sponsor. It should also be specified that these values are against the erythrocytic stages of *P. falciparum*. These IC_{50} values should be as follows:

Drug	IC_{50} values nM (ng/ml)	Reference
Atovaquone	0.04 to 9.6/55.7 (0.02 to 3.5/20.4)	Gay *et al.*, 1997, Am J Trop Med Hyg. 56: 315, Zalis *et al.*, 1998, Am. J. Trop. Med. Hyg. 58: 630, & Hudson et al., 1991, Drugs Expd. Clin. Res. 17: 427.
Folate free/low folate medium:		
Cycloguanil	0.3 to 1360 (0.08 to 342.7)	Ringwald et al., 1996, Am. J. Trop. Med. Hyg 55: 254, Watkins *et al.*, 1987, Am. J. Trop. Med. Hyg. 37: 273, & Khan et al., 1997, Trans. Roy. Soc. Trop. Med. Hyg. 91: 456.
Proguanil	3239 to 13233 (822.7 to 3361.2)	MALB 2001
Normal medium:		
Cycloguanil	17.6 to 5108 (4.4 to 1287.2)	Watkins *et al.*, 1984, Ann. Trop. Med. Parasitol. 78: 273.
Proguanil	2422 to 19243 (615.2 to 4887.7)	Watkins *et al.*, 1984, Ann. Trop. Med. Parasitol. 78: 273.
pCBG	11376 to 40662 (2411.7 to 8620.3)	Watkins *et al.*, 1984, Ann. Trop. Med. Parasitol. 78: 273.

The IC_{50} values for cycloguanil, proguanil, and pCBG varied from 17.6 to 5108 (i.e., 4.4 to 1287.2 ng/ml), 2422 to 19243 (i.e., 615.2 to 4887.7 ng/ml) and 11376 to 40662 (i.e., 2411.7 to

8620.3 ng/ml) nM respectively in normal medium. In p-aminobenzoic acid or folate free medium the cycloguanil IC_{50} values were 0.03 to 1360 nM (i.e., 0.007 to 342.7 ng/ml) and that of proguanil 3239 to 13233 nM (i.e., 822.7 to 3361.2 ng/ml).

3. It may be worthwhile to specify that the IC_{99} values against one of the strains (FCW-1) were shown to be 61 – 969 fold higher than the IC_{50} values. Such a variability could be due to different lots of the drug or different time testing.

4. The sponsor has stated that the ' [redacted] Although it is true that atovaquone in combination with proguanil exhibited synergistic activity *in vitro* against 3 strains of *P. falciparum*, the majority of the antiplasmodial activity is due to its major metabolite cycloguanil. The activity of the combination of atovaquone + cycloguanil varied from synergistic to weakly antagonistic against 5 different clones of *P. falciparum*. Therefore, the statement proposed by the sponsor should be modified to reflect the observations.

5. Exposure to atovaquone or proguanil alone *in vitro* or *in vivo* has been shown to induce resistance in *Plasmodium* species. The possibility of development of resistance after prolonged therapy with the combination of atovaquone + proguanil cannot be ruled out. Therefore, the following information should be stated in a resistance subsection within the [redacted] section of the label:

[redacted]

RECOMMENDATIONS:

This NDA is approvable with respect to microbiology pending an acceptable version of the label. The sponsor should be requested to conduct the following phase IV studies:

1. [redacted]

2. [redacted]

3. [redacted]

/S/
Shukal Bala
Microbiologist, HFD-590

CONCURRENCES:

HFD-590/Deputy Dir. /S/ Signature 6/11/99 Date
HFD-590/MicroTL /S/ Signature 6/4/99 Date

CC:
HFD-590/Original NDA # 21-078
HFD-590/Division File
HFD-590/MO/ MeyerhoffA
HFD-590/MO/ SacksL
HFD-590/Pharm
HFD-590/Chem
HFD-590/Review Micro
HFD-590/CSO/DempseyM