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PS-15: A POTENT, ORALLY ACTIVE ANTIMALARIAL FROM A NEW CLASS OF FOLIC ACID ANTAGONISTS

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Abstract. A new, orally-active inhibitor of dihydrofolic acid reductase (DHFR), PS-15 (N-(3-(2,4,5-trichlorophenoxy)propyloxy)-N'-(1-methylethyl)-imidocarbonimidicdiamide hydrochloride), has significant activity against drug-resistant *Plasmodium falciparum*. It is not cross-resistant with other inhibitors of DHFR (e.g., pyrimethamine and cycloguanil). Although it bears similarities to proguanil, PS-15 represents a new antifolate class of drugs that we have named oxyguanils or hydroxylamine-derived biguanides. This compound displays intrinsic antimalarial activity and also is metabolized in vivo to WR99210, an extremely active triazine inhibitor of DHFR. When tested in vitro against drug-resistant clones of *P. falciparum*, PS-15 was more active than proguanil, and the putative metabolite, WR99210, was more active than the proguanil metabolite cycloguanil. The drug is also more active as well as less toxic than proguanil when administered orally to mice infected with *P. berghei*. When administered orally to *Aotus* monkeys infected with multidrug-resistant *P. falciparum*, PS-15 was more active than either proguanil or WR99210. In 1973, WR99210 underwent clinical trials for safety and tolerance in volunteers. The trials showed gastrointestinal intolerance and limited bioavailability; further development of the drug was abandoned. Because PS-15 has intrinsic antimalarial activity, is not cross-resistant with other DHFR inhibitors, and can be metabolized to WR99210 in vivo, oral administration of this new drug should circumvent the shortcomings and retain the advantages found with both proguanil and WR99210.

Drug-resistant *Plasmodium falciparum* malaria will continue to be a serious clinical problem for the foreseeable future. At present, this problem can only be controlled by the periodic introduction of new antimalarial drugs from novel classes; PS-15 is such a new drug (Figure 1). It was designed to take advantage of the positive attributes of two old antimalarial drugs, proguanil and WR99210. Although never produced for clinical use, the antimalarial activity of WR99210 was first reported by Rieckmann in 1973 when in vitro inhibition studies with *P. falciparum* showed marked activity against both pyrimethamine-sensitive and pyrimethamine-resistant isolates.[1] Clinical pharmacology studies of WR99210 in humans, however, showed that doses of 200 mg five times a day for three days produced severe gastrointestinal symptoms and development of the drug was abandoned.[2]

Proguanil has remained an important antimalarial drug since it was first described in 1945.

ide proguanil to the triazine cycloguanil, also an inhibitor of dihydrofolic acid reductase (DHFR). Based on analogy, the biguanide precursor for the triazine WR99210 was designed and synthesized. This report summarizes the synthesis and results of antimalarial testing for this novel compound designated PS-15.

MATERIALS AND METHODS

Synthesis

The drug PS-15 was prepared as the hydrochloride hydrate from 3-(2,4,5-trichlorophenoxy)propyloxy amine hydrochloride and excess isopropyl dicyandiamide heated in ethanol at reflux. The propyloxyamine was synthesized in 58% yield by modification of the method of Mamalis and Outred (U.S. Patent 3723429, March 27, 1973). Elemental analysis, infrared, and nuclear magnetic resonance (NMR) proton spectra con[illegible] the structure. The dicyandiamide was

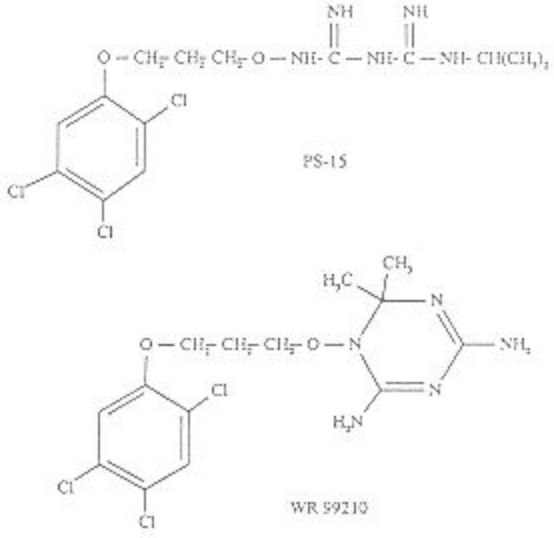

FIGURE 1. Chemical structures of PS-15 and WR 99210.

of Curd and others.[3]. The reaction product was recrystallized from ethyl acetate to give 15% PS-15 as the hydrochloride monohydrate with a melting point of 102–106°C. The structure was confirmed by elemental, infrared, and NMR (proton and carbon) analyses. The calculated elemental composition for PS-15 ($C_{14}H_{20}Cl_3N_5O_2 \cdot HCl \cdot H_2O$) is C: 37.27; H: 5.14; N: 15.52; analysis found C: 37.44; H: 5.05; N: 15.29.

In vitro activity

In vitro tests of activity for PS-15, WR99210, proguanil, and cycloguanil were performed against *P. falciparum* using modifications[4] of the semiautomated microdilution technique of Desjardins and others.[5] Antimalarial activity in this system was assessed by inhibition of radiolabeled hypoxanthine incorporation into parasites by graded concentrations of drugs during incubation for 66 hr in culture medium containing physiologic concentrations of folic acid. Three parasite clones were studied: the Indochina W-2 clone (resistant to chloroquine, quinine, sulfadoxine, and pyrimethamine), the Sierra Leone D-6 clone (resistant to mefloquine), and the Colombian FCB clone (resistant to cycloguanil).[6, 7] All drugs studied, as well as control drugs, were tested in duplicate. In this test system, the results are expressed by estimating the concentrations of drugs that produced 50% inhibition of radiolabel incorporation (IC_{50}).

Tests for drug interactions between PS-15 and other selected antimalarial drugs were done by combining PS-15 with another drug in fixed ratios proportional to their respective IC_{50}s and serially diluting the combinations for assessment of in vitro activity as described above. The estimated IC_{50}s of PS-15, the drug being combined with it, as well as all fixed ratios studied, were normalized to unity. From these data, isobolograms were constructed to visually demonstrate the degree of interaction.[8] Synergy is demonstrated when the data fall below an imaginary line connecting the IC_{50} of PS-15 with the IC_{50} of the other antimalarial drug.

In vivo activity

In vivo tests of antimalarial activity were conducted in mice infected with drug-sensitive *P. berghei* KBG 173 by administering graded doses of drug to groups of five CD-1 Swiss mice three days after being injected with lethal inocula of parasites.[9] For subcutaneous administration, the drugs were suspended or dissolved in peanut oil; for oral administration, the drugs were suspended in hydroxyethylcellulose and Tween 80. Control mice not receiving drug treatment died of their infections within 6–7 days; mice receiving curative doses of drugs survived for at least 60 days. The results of these tests are expressed by log-probit estimations of the dose of drug that cured 50% of the mice (CD_{50}).

Tests of in vivo antimalarial activity were also conducted in Panamanian owl monkeys (*Aotus lemurinus lemurinus*) infected with multidrug-resistant Vietnamese strains of *P. falciparum*. Proguanil, cycloguanil and PS-15 were tested against the Vietnam Smith/RE strain and WR99210 against the Vietnam Monterey strain. Both strains are solidly resistant to chloroquine, quinine, and pyrimethamine.[10] Drugs were suspended in methylcellulose and administered in graded doses orally once a day for three days to groups of monkeys with established infections. Serial blood films were obtained to determine parasite suppression or clearance. If parasites cleared and there was no evidence of recrudescence within 100 days, the infections were considered cured. The results of these studies are expressed by log-probit estimations of the CD_{50} expressed in total dose of drug administered during the three days.

TABLE 1

Comparative antimalarial efficacy of PS-15 against drug-resistant clones of Plasmodium falciparum *in vitro**

Drug	Clone		
	Indochina W-2	Sierra Leone D-6	Colombian FCB
PS-15	664	19.4	541
WR99210	0.081	0.044	0.088
Proguanil	3,205	251	1,246
Cycloguanil	0.56	0.03	13.4

* Values are 50% inhibitory concentrations (ng/ml).

RESULTS

The results of the in vitro sensitivity tests against *P. falciparum* are shown in Table 1. The drug PS-15 was more active than proguanil against all three clones of parasites studied. In addition, the putative metabolite WR99210 was more active than cycloguanil, except against the mefloquine-resistant D-6 clone.

In vitro tests for drug interaction (Figure 2) showed PS-15 to exhibit synergy with sulfamethoxazole and atovaquone (BW566C), a new hydroxynaphthoquinone being evaluated for antimalarial efficacy by Wellcome Research Laboratories, Beckenham, UK (Hutchinson DBA, unpublished data).

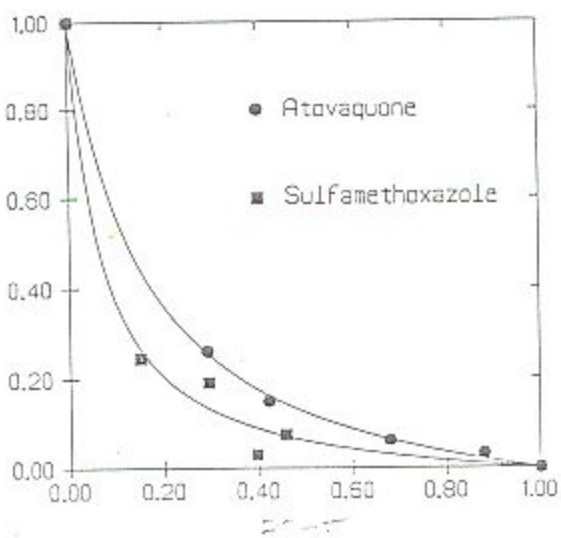

FIGURE 2. Isobologram of *Plasmodium falciparum* in vitro activity, showing PS-15 in combination with atovaquone or sulfamethoxazole. Both drugs exhibited synergy with PS-15.

TABLE 2

Comparative antimalarial efficacy of PS-15 in mice infected with Plasmodium berghei

Drug	Route of administration	50% curing dose (mg/kg)
PS-15	Subcutaneous	154
	Oral	345
WR99210	Subcutaneous	462
	Oral	>640
Proguanil	Subcutaneous	Toxic >160
	Oral	Toxic >160
Cycloguanil	Subcutaneous	165
	Oral	>640

The results of the in vivo studies in mice are summarized in Table 2. When administered orally in doses up to 640 mg/kg, WR99210, proguanil, and cycloguanil showed no activity, whereas PS-15 was curative (CD_{50} = 345 mg/kg). When administered subcutaneously, proguanil was toxic, but PS-15, cycloguanil, and WR99210 were all active, with PS-15 being the most active (CD_{50} = 154 mg/kg).

In vivo studies in monkeys confirmed these findings; the results are shown in Table 3. When administered orally, neither proguanil nor cycloguanil were curative at the doses tested. However, both PS-15 and WR99210 were extremely active, with PS-15 being more active than WR99210.

DISCUSSION

During World War II, British investigators synthesized a series of new substituted biguanides that were active against both blood and exoerythrocytic forms of malaria parasites in chicks.[1] The most active of this series was proguanil. Despite the demonstration of in vivo antimalarial activity of this compound by Curd and others,[1] in vitro studies showed that the growth of *P. gallinaceum* in tissue cultures was not in-

TABLE 3

Comparative antimalarial efficacy of PS-15 in monkeys infected with Plasmodium falciparum

Drug	Route of administration	50% curing dose (mg/kg)
PS-15	Oral	15
WR99210	Oral	35
Proguanil	Oral	>150
Cycloguanil	Oral	>150

hibited at concentrations of drug thought to be present in human plasma.[12] In contrast, serum taken from a patient being treated with proguanil arrested the in vitro development of *P. falciparum*.[13] Experimental studies of this phenomenon using *P. cynomolgi*–infected blood confirmed the lack of in vitro activity of proguanil itself, and substantiated the activity of serum taken from monkeys treated with proguanil when the serum was added to malaria cultures.[14] Additional studies showed that proguanil could be activated, not only by administration to animals, but also by incubation with minced liver.[15] These results led the investigators to conclude that proguanil itself was not active against malaria, but that a metabolite was responsible for its antimalarial effect.

Because proguanil was structurally similar to pyrimidine derivatives that had been shown to inhibit the growth of *Lactobacillus casei* in the absence of purines, Falco and others tested proguanil and found that it also inhibited growth of *L. casei*, but only at high concentrations.[16] Like the pyrimidines, proguanil inhibition of growth could be reversed by either pteroylglutamic acid or purines.

The explanation for the apparent discrepancies between potent in vivo antimalarial activity, minimal in vitro inhibition of *L. casei* growth, and the apparent absence of in vitro antimalarial activity, began to unfold two years later when metabolic studies with proguanil were reported. The initial studies suggested the presence of two metabolites, as well as unchanged drug, in the livers of rhesus monkeys and in the urine of humans and monkeys to whom proguanil had been administered.[17] One metabolite was thought to be N1-*p*-chlorophenylbiguanide and the second a dihydrotriazine. The correct structure for the triazine was described by Carrington and others shortly thereafter, and the compound is now called cycloguanil.[18] This triazine was later isolated from the urine of rabbits after administration of proguanil and was reported to be ten times more active than proguanil itself against *P. gallinaceum* in chicks.[19] This was considered to be convincing evidence that the antimalarial activity of proguanil resulted solely from conversion of proguanil to cycloguanil.

This conclusion, however, was questioned when Schmidt and others compared the efficacy of proguanil with cycloguanil in rhesus monkeys and found proguanil to be more potent than cycloguanil.[20] This study of antimalarial efficacy in monkeys was followed by a series of metabolic studies in which Smith and others measured the concentrations of proguanil and its metabolites in plasma and urine from rhesus monkeys by a combination of chemical assay and microbiologic assay for antifolate activity.[21] These studies showed that the amount of cycloguanil produced after administration of proguanil was always less than the amount of cycloguanil measured after administration of an equal dose of cycloguanil. Based on the superior activity of proguanil in monkeys observed by Schmidt and others, Smith and others concluded, therefore, that the antimalarial activity of proguanil must be due in part to antimalarial activity of the parent drug.

This position was supported by in vitro studies with *P. falciparum* in which the media contained only physiologic concentrations of folic acid and *p*-aminobenzoic acid (PABA). Previous studies conducted by Milhous and others showed that the excess exogenous folate found in normal RPMI 1640 culture media markedly antagonized the activity of both DHFR inhibitors and PABA antagonists.[4] The present studies, which used media containing physiologic concentrations of folic acid and PABA, showed that not only that proguanil has intrinsic antimalarial activity, but that inhibitory concentrations could probably be achieved with ordinary human doses due to accumulation of proguanil in red blood cells. In this in vitro system, proguanil also exhibited synergy with sulfonamides and activity was reversed with folinic acid (Milhous WK, unpublished data).

The drug WR99210 (also known as BRL 6231) was selected for development by the U.S. Army Antimalarial Drug Development Program from a series of N-substituted triazines inhibitors of DHFR with antimalarial activity.[22] This compound was initially selected because it was extremely active in vitro and because it showed no evidence of cross-resistance with pyrimethamine.[1] Further studies in vivo confirmed the lack of cross-resistance with pyrimethamine, and showed only negligible cross-resistance with cycloguanil.[23] The compound WR99210 also showed evidence of causal prophylactic activity in both mice challenged with *P. berghei* and monkeys challenged with *P. cynomolgi*. Further studies in *P. berghei*–infected mice showed that although resistance to WR99210 could be induced by increasing drug pressure, the rate of devel-

opment and degree of resistance was less than with other inhibitors of DHFR, notably pyrimethamine and cycloguanil.[24]

Preclinical studies with WR99210 were done in 1972. The acute oral and intraperitoneal 50% lethal doses were 1,980 mg/kg and 84 mg/kg, respectively, in rats and 3,510 mg/kg and 108 mg/kg, respectively, in mice. Repeated oral doses of 50 mg/kg of WR99210 for 28 consecutive days were well-tolerated in rats, but repeated oral doses of 100, 200, or 400 mg/kg produced dose-related gastrointestinal findings. In dogs, repeated doses of 5 mg/kg/day were well-tolerated, but doses of 10 mg/kg/day produced gastrointestinal symptoms and findings; doses of 25 mg/kg/day were lethal. A repeat study in beagles confirmed the presence of gastrointestinal intolerance at doses of 15 mg/kg/day, but these findings could be abrogated by concurrent administration of folinic acid in doses of 0.3 mg/kg/day.

Phase I tolerance studies were initiated with WR99210 in 1973 using a double-blinded rising-dose design. Doses of 200 mg or greater per day for three days produced mild intermittent anorexia, nausea and abdominal pain; divided doses of 1 g/day for three days produced severe nausea, vomiting, and diarrhea that persisted for two days after drug administration was stopped. A subsequent pharmacokinetic study with single doses of 50 mg or 200 mg of WR99210 was performed using a bioassay for DHFR inhibition with *Streptococcus fecalis*. Only trace amounts of DHFR inhibition were detected in plasma from two volunteers receiving single 50-mg doses; no activity was detected in volunteers receiving single 200-mg doses. Because of the gastrointestinal intolerance and poor bioavailability, clinical development of WR99210 by the Army Antimalarial Drug Program was abandoned.

In 1985, PS-15 was conceived as a member of a new class of drugs with antimalarial activity by analogy to proguanil. It was reasoned that this drug might not only have inherent activity, but could also be orally bioavailable as a lipophilic biguanide and metabolically converted to WR99210 by the same microsomal mixed function oxidase that converts proguanil to cycloguanil.[25] To evaluate this possibility, the drug was synthesized and tested in both in vitro and in vivo test systems. Preliminary studies in monkeys demonstrated the presence of WR99210 in plasma from animals receiving PS-15 (Edstein MD, unpublished data).

When administered orally to mice with *P. berghei* infections, PS-15 was found to be more active than proguanil, cycloguanil, or WR99210. These findings were confirmed when the same comparisons were made in *Aotus* monkeys infected with *P. falciparum*. When administered subcutaneously to infected mice, PS-15 was as active as cycloguanil and showed no evidence of toxicity. In contrast, proguanil in doses greater than 160 mg/kg was lethal to some mice.

Studies of antimalarial activity in vitro showed that PS-15 was more active than proguanil against all three *P. falciparum* clones tested. Although in vitro conversion to WR99210 by infected red blood cells cannot be excluded, this seems highly unlikely based on the known method of conversion of proguanil to cycloguanil. The drug PS-15 also potentiated the activity of sulfamethoxazole in vitro, suggesting that it is an inhibitor of DHFR itself. Such inhibitors are usually associated with rapid induction of resistance. However, PS-15 is converted to WR99210, a drug to which resistance develops slowly in animal models. Furthermore, based on the in vitro evidence for synergy with sulfamethoxazole and atovaquone, concurrent use of PS-15 and one of these drugs should not only increase the potency of both compounds, but also delay the development of resistance to the combination.

In summary, PS-15 represents a new class of folic acid antagonists that demonstrates surprising activity when tested in vitro against both drug-sensitive and -resistant *P. falciparum* as well as when tested in vivo in *Aotus* monkeys. Both PS-15 and its metabolite WR99210 are active, and thus achieve the same pattern of activity as proguanil and its metabolite cycloguanil. However, PS-15 was more active than proguanil and WR99210 was more active than cycloguanil. Further studies with PS-15 are planned to determine if this new drug class will be as safe and well-tolerated as proguanil, and to determine whether the problem of the gastrointestinal intolerance, which is associated with WR99210 administration to humans, has been eliminated.

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REFERENCES

1. Rieckmann KH, 1973. The in vitro activity of experimental antimalarial compounds against strains of P. falciparum with varying degrees of sensitivity to pyrimethamine and chloroquine. Chemotherapy of Malaria and Resistance to Antimalarials. *World Health Organ Tech Rep Ser 529:* 58.
2. Canfield CJ, 1986. New antimalarials under development. Bruce-Chwatt, LJ, ed. *Chemotherapy of Malaria.* Revised second edition. Geneva: World Health Organization Monograph Series. No. 27, 99–100.
3. Curd JA, Hendry TS, Kenny TS, Murray AG, Rose FL, 1948. Synthetic antimalarials. Part XXVII: An alternative route to N¹-aryl-N⁵-alkyldiguanides. *J Chem Soc:* 1630–1635.
4. Milhous WK, Wetherly NF, Bowdre JH, Desjardins RE, 1985. In vitro activities and mechanisms of resistance to antifol antimalarial drugs. *Antimicrob Agents Chemother 27:* 525–530.
5. Desjardins RE, Canfield CJ, Haynes JD, Chulay JD, 1979. Quantitative assessment of antimalarial activity in vitro by a semiautomated microdilution technique. *Antimicrob Agents Chemother 16:* 710–718.
6. Oduola AMJ, Wetherly NF, Bodwre JH, Desjardins RE, 1988. Plasmodium falciparum: cloning by single-erythrocyte micromanipulation and heterogeneity in vitro. *Exp Parasitol 66:* 86–95.
7. Peterson D, Milhous WR, Wellems TE, 1990. Molecular basis of differential resistance to cycloguanil and pyrimethamine in Plasmodium falciparum malaria. *Proc Natl Acad Sci USA 87:* 3018–3022.
8. Rolo UM, 1955. The mode of action of sulphonamides, proguanil and pyrimethamine on Plasmodium gallinaceum. *Br J Pharmacol 10:* 208–214.
9. Osdene TS, Russell PB, Rane L, 1967. 2,4,7-triamino-6-ortho-substituted arylpteridines. *J Med Chem 10:* 431–434.
10. Schmidt LH, 1973. Infections with Plasmodium falciparum and Plasmodium vivax in the owl monkey - model systems for basic biological and chemotherapeutic studies. *Trans R Soc Trop Med Hyg 67:* 446–474.
11. Curd FHS, Davey DG, Rose FL, 1945. Studies on synthetic antimalarial drugs. X. Some biguanide derivatives as new types of antimalarial substances with both therapeutic and causal prophylactic activity. *Ann Trop Med Parasitol 39:* 208–216.
12. Tonkin IM, 1946. The testing of drugs against exoerythrocytic forms of P. gallinaceum in tissue culture. *Br J Pharmacol 1:* 163–173.
13. Black RH, 1946. The effect of anti-malarial drugs on Plasmodium falciparum (New Guinea strains) developing in vitro. *Trans R Soc Trop Med Hyg 40:* 163–170.
14. Hawking F, 1947. Activation of paludrine in vitro. *Nature 159:* 409.
15. Hawking F, Perry WL, 1948. Activation of paludrine. *Br J Pharmacol 3:* 320–325.
16. Falco EA, Hitchings, GH, Russell PB, VanderWerff H, 1949. Antimalarials as antagonists of purines and pteroylglutamic acid. *Nature 164:* 107–108.
17. Crounse NN, 1951. Isolation and identification of a metabolite of chlorguanide. *J Org Chem 16:* 492–500.
18. Carrington HC, Crowther AF, Davey DG, Levi AA, Rose FL, 1951. A metabolite of paludrine with high antimalarial activity. *Nature 168:* 1080.
19. Crowther AF, Levi AA, 1953. Proguanil - The isolation of a metabolite with high antimalarial activity. *Br J Pharmacol 8:* 93–97.
20. Schmidt LH, Loo TL, Fradkin R, Hughes HB, 1952. Antimalarial activities of triazine metabolites of chlorguanide and dichlorguanide. *Proc Soc Exp Biol Med 80:* 367–370.
21. Smith CC, Ihrig J, Menne R, 1961. Antimalarial activity and metabolism of biguanides. I. Metabolism of chlorguanide and chlorguanide triazine in rhesus monkeys and man. *Am J Trop Med Hyg 10:* 694–703.
22. Canfield CJ, Rozman RS, 1974. Clinical testing of new antimalarial compounds. *Bull World Health Organ 50:* 203–212.
23. Knight DJ, Manalis P, 1982. The antimalarial activity of N-benzyl-oxydihydrotriazines. III. The activity of 4,6-diamino-1,2-dihydro-2,2-dimethyl-1-(2,4,5-trichloropropyloxy)-1,3,5-triazine hydrobromide (BRL 51084) and hydrochloride (BRL6231). *Ann Trop Med Parasitol 76:* 1–7.
24. Knight DJ, Williams P, 1982. The antimalarial activity of N-benzyl-oxydihydrotriazines. IV. The development of resistance to BRL 6231 (4,6-diamino-1,2-dihydro-2,2-dimethyl-1-(2,4,5-trichloropropyloxy)-1,3,5-triazine hydrochloride) by Plasmodium berghei. *Ann Trop Med Parasitol 76:* 9–14.
25. Armstrong VL, 1972. *Metabolism of Chlorguanide, a Unique Mixed Function Oxidase Substrate.* Ph.D Thesis, Department of Environmental Health, University Cincinnati College of Medicine, Cincinnati, OH.