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SHORT COMMUNICATION

Studies on the Mechanism of Antimalarial Action of a Novel Arylene Bis(methylketone)

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ABSTRACT. 2-Amino-4-(3,5-diacetylphenyl)amino-1,6-dimethylpyrimidinium chloride (CNI-H0294) is a novel arylene bis(methylketone) compound that displays antimalarial activity against chloroquine- and pyrimethamine-resistant *Plasmodium falciparum* clones. The compound has been found to be concentrated into infected erythrocytes, with 80–179 μM accumulated when parasites were cultured in the presence of 1.0 μM CNI-H0294. Uninfected erythrocytes, in contrast, only accumulated 2.5–3.4 μM CNI-H0294 under identical conditions. Using postmitochondrial supernatants from a number of parasite clones, the compound was found to inhibit dihydrofolate reductase (EC 1.5.1.3) activity with an IC_{50} of 243–483 μM. Thus, while CNI-H0294 is not a powerful inhibitor of plasmodial dihydrofolate reductase, the accumulation of the compound into infected erythrocytes, when correlated to the external ED_{50} concentration against parasite growth *in vitro*, reaches concentrations sufficient to inhibit the malarial enzyme. BIOCHEM PHARMACOL **54**;6:739–742, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. malaria; drug accumulation; dihydrofolate reductase; arylene bis(methylketone)s

Malaria remains one of the world's largest causes of mortality and morbidity, with an estimated 2.1 million fatalities and up to 500 million infections annually [1]. The situation regarding the control and treatment of malaria has progressively worsened with the spread of insecticide-resistant mosquito vectors and drug-resistant parasites [1]. In the face of these events, the development of novel, nontoxic antimalarials is of crucial importance. 2-Amino-4-(3,5-diacetylphenyl)amino-1,6-dimethylpyrimidiniumchloride (CNI-H0294)† (see ref. [2] for the structure) is a novel arylene bis(methylketone) compound that was originally synthesized as an intermediate in the construction of an antitrypanosomal bis-guanylhydrazone analogue of quinapyramine (data not shown). Subsequently, CNI-H0294 was found to have antihuman immunodeficiency virus activity as measured by virus replication in nondividing host cells, such as macrophages [3]. The mechanism of this antiviral activity was clearly due to the prevention of the translocation of the viral nucleic acid to the nucleus of the host cell. More recently, CNI-H0294 was found to display considerable antimalarial activity *in vitro* against *Plasmodium falciparum* and *in vivo* against *P. berghei* [2]. Moreover, clones of *P. falciparum* resistant to chloroquine and/or pyrimethamine (PYR) were fully sensitive to CNI-H0294. The compound was also found to be nontoxic in mice [2] and to have rapid plasma kinetics [4].

While the mechanism of antimalarial activity remained unknown, it was presumed to be different from that of the antiviral activity based on structure-activity relationships of several analogues [2]. In this paper, we demonstrate that CNI-H0294 accumulates 80–179-fold into malaria-infected erythrocytes. In addition, the drug was found to display activity against the plasmodial dihydrofolate reductase (DHFR; EC 1.5.1.3) at concentrations achievable in infected erythrocytes. This enzyme inhibition thus represents one possible mechanism by which CNI-H0294 exerts its antimalarial effect.

MATERIALS AND METHODS

P. falciparum clones D10, Dd2, HB3, and FCR-3 were cultured in human A+ erythrocytes by the method of Trager and Jensen [5]. Details of the ED_{50} values for chloroquine and PYR for these clones can be obtained from ref. [2]. RPMI 1640 medium was acquired from Gibco (Gaithersburg, MD, USA) and human A+ erythrocytes and fresh frozen plasma from Long Island Blood Services (Melville, NY, USA). PYR, heptane sulfonate, bovine DHFR, and saponin were obtained from Sigma (St. Louis, MO, USA), tetramethylammonium chloride and phosphoric acid from Aldrich (Milwaukee, WI, USA), and HPLC grade acetonitrile from Fisher (Fairlawn, NJ, USA). CNI-H0294 was synthesized in our laboratories as previously described [2].

To measure CNI-H0294 accumulation into infected and uninfected erythrocytes, duplicate 20-ml cultures were made of distilled water, complete malaria medium, com-

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† *Abbreviations:* DHFR, dihydrofolate reductase; HPLC, high performance liquid chromatography; PYR, pyrimethamine.

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TABLE 1. The accumulation of CNI-H0294 by infected erythrocytes

Sample	RBC (× 10^9) Start	RBC (× 10^9) Final	Parasitemia (%) Start	Parasitemia (%) Final	nmol CNI-H0294 in supernatant	nmol CNI-H0294 in RBC	nmol CNI-H0294 per 10^9 RBC	nmol CNI-H0294 per 10^9 infected RBC
[a]Medium—	—		—	—	17.78	—	—	—
[a]RBC	9.2	9.0	—	—	15.71	3.36	0.37	—
[a]FCR3-RBC	8.6	6.2	3.1	5.7	12.35	5.42	0.87	8.76
[a]HB3-RBC	5.6	5.2	5.9	10.5	10.42	6.95	1.34	9.24
[b]Medium	—	—	—	—	22.73	—	—	—
[b]RBC	14.6	6.6	—	—	19.69	1.82	0.27	—
[b]HB3-RBC	9.8	3.8	8.1	11.2	14.23	7.24	1.91	14.59
[b]FCR3-RBC	9.4	4.4	5.4	10.9	14.98	6.24	1.42	10.50
[b]D10-RBC	9.6	4.2	5.3	9.4	11.80	4.77	1.14	9.16
[b]Dd2-RBC	7.8	3.6	8.7	7.7	12.50	6.36	1.77	19.51

a and b represents a group wherein each experiment was made with the same stock of CNI-H0294. Thus, the total amount of CNI-H0294 in group a was 18.00 ± 0.74 nmol and in group b was 20.39 ± 2.26 nmol.

plete malaria medium plus human erythrocytes at 5% hematocrit, or complete medium plus malaria-infected erythrocytes at 5% hematocrit. An aliquot of each culture was taken to measure parasitemia and erythrocyte number. CNI-H0294 was added to 1 μM to one set of the cultures and mixed well before incubation at 37° for 48 hr. The 48-hr incubation was chosen to duplicate that used for the *in vitro* ED_{50} assays [2]. The cultures were then gently resuspended, and an aliquot was taken to measure parasitemia and erythrocyte number. Each culture was centrifuged at 3000 × *g* in a clinical centrifuge for 10 min, and the supernatant and erythrocyte pellet were separated. Six ml of 10 mM tetramethylammonium chloride, 10 mM heptane sulfonate, 4.2 mM H_3PO_4, 95% CH_3CN, 5% H_2O were added to each erythrocyte pellet (see Table 1 for the cell numbers in each pellet) before sonication on ice. The disrupted cells were centrifuged at 3000 × rpm in a clinical centrifuge to pellet the precipitated material. The supernatant was lyophilized and resuspended in 100 μl of 10 mM tetramethylammonium chloride, 10 mM heptane sulfonate, 4.2 mM H_3PO_4, H_2O before analysis by HPLC as described [4]. Two ml of 100 mM tetramethylammonium chloride, 100 mM heptane sulfonate, 42 mM H_3PO_4, H_2O was added to 20 ml of the culture supernatants before solid-phase extraction and HPLC analysis as described [4].

For measuring dihydrofolate reductase inhibition, cultures of *P. falciparum* were allowed to become asynchronous and grown to 5–10% parasitemia at 5% hematocrit. When the cultures were predominantly, but not exclusively, late trophozoites/schizonts, the cultures were resuspended and saponin lysed, and the parasites were isolated and resuspended in ice cold 10 mM sodium phosphate, pH 7.4, 1 mM EDTA, 1 mM 2-mercaptoethanol, 1.8 mg/ml benzamidine, 50 μg/ml soybean trypsin inhibitor, 50 μg/ml aprotinin. The parasites were then gently homogenized with a Dounce homogenizer kept on ice, centrifuged at 14,000 × *g* for 10 min, and the supernatant was immediately used as an enzyme source for assaying DHFR activity, using 100 μM dihydrofolate and 50 μM NADPH, as described [6]. At least five drug concentrations were tested per experiment, and the inhibition of activity was utilized to calculate an IC_{50} value with the Enzfitter software (Cambridge Biosoft; Cambridge, UK) programmed with the Chou equation [7].

RESULTS AND DISCUSSION

In trying to determine a possible mechanism for the antimalarial activity of CNI-H0294, it was first important to determine to what extent the compound penetrates into infected and uninfected erythrocytes. The cells were grown, under identical conditions to the *in vitro* ED_{50} assays [2], in the presence or absence of subtherapeutic concentrations of CNI-H0294 (0.25–0.50 of the ED_{50}), before being extracted and analyzed by HPLC. In test extractions, the recovery of CNI-H0294 from the culture medium was found to be very high (86.07 ± 14.88%, $n = 3$), while the recovery from the erythrocytes was low but consistent (24.97 ± 10.68%, $n = 8$). As seen in Table 1, infected erythrocytes concentrated CNI-H0294 from 1 μM external to 8.76–19.51 nmol/10^9 infected erythrocytes. As 1.0 ml of packed erythrocytes was found to contain 9.2 ± 2.5 × 10^9 ($n = 8$) cells, this amount of drug represented an internal CNI-H0294 concentration of 80.37–178.99 μM. In contrast, uninfected erythrocytes were found to contain 0.27–0.37 nmol/10^9 cells, which is equivalent to an internal concentration of 2.48–3.39 μM. No metabolites of CNI-H0294, as described in ref [4], were found in either the culture supernatant or the cell pellet.

One feature of these accumulation experiments was the relationship between starting parasitemia and drug concentration in infected erythrocytes. CNI-H0294 levels in the culture supernatants decreased in direct inverse proportion to the starting parasitemia ($n = 5$, $R = 0.82$), while the levels of drug recovered from the infected erythrocytes directly increased ($n = 5$, $R = 0.94$). At this point, it is impossible to clearly distinguish between active uptake of CNI-H0294 by infected erythrocytes or the parasites themselves, and whether infection is associated with an increased permeability of erythrocytes with respect to the

TABLE 2. The inhibition of DHFR by CNI-H0294 and PYR

	DHFR inhibition (IC_{50})		Growth inhibition (ED_{50})	
Enzyme source	CNI-H0294 (μM)	PYR (nM)	CNI-H0294 (μM)	PYR (μM)
P. falciparum				
D10	429 ± 30 (4)	1690 (1)	4.0 ± 0.4	170 ± 25
Dd2	483 ± 52 (5)	1470 (1)	3.5 ± 0.1	100 ± 10
FCR-3	411 ± 28 (4)	0.36 (1)	3.1 ± 0.3	0.04 ± 0.01
HB3	243 ± 27 (5)	24.0 (1)	1.8 ± 0.3	9.0 ± 2.8
Bovine liver	733 ± 10 (3)	266 ± 33 (3)		

The IC_{50} values for both compounds against plasmodial or bovine DHFR were determined as described under "Materials and Methods." For comparison, the concentration of CNI-H0294 or PYR necessary to inhibit the growth of each *P. falciparum* clone in culture (ED_{50}) has been included from ref [2]. Where appropriate, the values are ± standard deviation, and the replicate number is in parentheses.

compound. However, the magnitude of the concentration effect would suggest that some parasite- or infected erythrocyte-specific uptake system is the more likely possibility. It should also be noted that incubation of uninfected erythrocytes in conditioned medium taken from infected erythrocyte cultures had no effect on the accumulation of CNI-H0294 by the uninfected cells. Thus, the pH change produced by parasite growth, or the presence of other parasite products in the culture supernatant, does not alter the permeability of uninfected erythrocytes to the drug.

With CNI-H0294 being concentrated over 100-fold from external concentrations, it becomes more difficult to determine a single, specific mechanism of action. Other antiparasitics that can reach similar internal concentrations, such as aromatic diamidines or suramin, have been shown to inhibit a large number of cellular processes [8]. Nevertheless, it is important to delineate which biochemical activities can be inhibited by achievable concentrations of CNI-H0294 to aid in the design of improved analogues. As CNI-H0294 displays some structural similarity to the DHFR inhibitors PYR, cycloguanil, and trimethoprim, it was possible that the compound has activity against the malarial DHFR. Using *P. falciparum* postmitochondrial supernatants as the source of parasite DHFR, CNI-H0294 was found to inhibit the enzyme (Table 2), with an IC_{50} of 242.56–482.86 μM. Moreover, there was a clone-specific, direct relationship between the DHFR IC_{50} and the corresponding ED_{50} value for CNI-H0294 inhibition of growth *in vitro* ($n = 4$, $R = 0.90$). In tests against commercial, bovine DHFR, CNI-H0294 had an IC_{50} of 732.92 μM, giving a therapeutic index of 1.53–3.04 for the clones studied. CNI-H1194 (see ref [2] for the structure), which is 3–4-fold less effective than CNI-H0294 in the ED_{50} assay [2], had an IC_{50} of 622 ± 10 μM ($n = 3$) against parasite DHFR, while CNI-H1894, which is ineffective in the ED_{50} assay, had no effect on parasite DHFR up to 3.0 mM ($n = 3$).

In contrast, PYR inhibited the plasmodial DHFR with an IC_{50} ranging from 0.36 to 1691.30 nM, depending on the resistance status of the clone (Table 2). Once again, there was a direct relationship with the corresponding ED_{50} value ($n = 4$, $R = 0.96$). With an IC_{50} of 265.67 nM against bovine DHFR, PYR had a therapeutic index of 0.15–737.69, depending on the parasite clone. It is clear that PYR is a more efficient inhibitor of both plasmodial and mammalian DHFR, but that clones with very high PYR resistance contain DHFR sensitive to CNI-H0294. It is also interesting to note that, for CNI-H0294, the DHFR IC_{50} was 128.13 ± 14.10 fold higher than the corresponding ED_{50} value, while the same ratio for PYR was 0.0091 ± 0.0049. These values provide further evidence that CNI-H0294 is concentrated against a gradient, and that PYR poorly penetrates the infected erythrocyte.

At the external ED_{50} concentrations of CNI-H0294, HB3-infected erythrocytes would be expected to contain 145–322 μM internal drug, FCR-3-infected erythrocytes 249–555 μM, Dd2-infected cells 281–626 μM, and D10-infected cells 321–716 μM. These calculated ranges cover the DHFR IC_{50} for each of the clones used. In addition, the measured CNI-H0294 may be located in the parasite itself, rather than the entire infected erythrocyte, which would entail a decrease in volume and a corresponding increase in the concentration of CNI-H0294 seen by the plasmodial DHFR. However, as mentioned above, drugs that accumulate to high internal concentration are likely to affect numerous cellular processes. At present, we are investigating whether CNI-H0294 inhibits other plasmodial biochemical pathways.

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Refrences

1. World Health Organization, *The World Health Report 1996: fighting disease, fostering development.* World Health Organization, Geneva, 1996.
2. Berger BJ, Paciorkowski A, Suskin M, Dai WW, Cerami A and Ulrich P, Antimalarial activity of novel arylene bis(methylketone) compounds. *J Infect Dis* **174:** 659–662, 1996.
3. Dubrovsky L, Ulrich P, Nuovo GJ, Manogue KR, Cerami A and Bukrinsky M, Nuclear localization signal of HIV-1 as a novel target for therapeutic intervention. *Mol Med* **1:** 217–230, 1995.
4. Berger BJ, Suskin M, Dai WW, Cerami A and Ulrich P, Studies on the pharmacological properties of novel arylene

bis(methylketone) compounds using solid-phase extraction and high-performance liquid chromatography. *J Chromatogr B* **691:** 433–440, 1997.
5. Trager W and Jensen JB, Human malaria parasites in continuous culture. *Science* **193:** 6673–6675, 1976.
6. Chen GX, Mueller C, Wendlinger M and Zolg JW, Kinetic and molecular properties of the dihydrofolate reductase from pyrimethamine-sensitive and pyrimethamine-resistant clones of the human malaria parasite *Plasmodium falciparum*. *Mol Pharmacol* **31:** 430–437, 1987.
7. Chou TC, Derivation and properties of Michaelis-Menton and Hill type equations for reference ligands. *J Theor Biol* **39:** 253–276, 1976.
8. Wang CC, Molecular mechanisms and therapeutic approaches to the treatment of African trypanosomiasis. *Annu Rev Pharmacol Toxicol* **35:** 93–127, 1995.