0026-895X/01/5905-1298–1306$3.00
MOLECULAR PHARMACOLOGY
Copyright © 2001 The American Society for Pharmacology and Experimental Therapeutics
Mol Pharmacol 59:1298–1306, 2001

Vol. 59, No. 5
651/900571
Printed in U.S.A.

Diamidine Compounds: Selective Uptake and Targeting in *Plasmodium falciparum*

ANDREW M. W. STEAD, PATRICK G. BRAY, I. GEOFFREY EDWARDS, HARRY P. DEKONING, BARRY C. ELFORD, PAUL A. STOCKS, and STEPHEN A. WARD

Department of Pharmacology and Therapeutics, The University of Liverpool, Liverpool, United Kingdom (A.M.W.S., P.G.B., I.G.E., P.A.S., S.A.W.); Division of Infection of Immunity, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow, United Kingdom (H.P.D.); and Molecular Parasitology Group, Institute of Molecular Medicine, Oxford, United Kingdom (B.C.E.)

Received October 19, 2000; accepted February 2, 2001

This paper is available online at http://molpharm.aspetjournals.org

ABSTRACT

Extensive drug resistance in *Plasmodium falciparum* emphasizes the urgent requirement for novel antimalarial agents. Here we report potent antimalarial activity of a number of diamidine compounds. The lead compound pentamidine is concentrated 500-fold by erythrocytes infected with *P. falciparum.* Pentamidine accumulation can be blocked by inhibitors of hemoglobin digestion, suggesting that the drug binds to ferriprotoporphyrin IX (FPIX). All of the compounds bound to FPIX in vitro and inhibited the formation of hemozoin. Furthermore, inhibitors of hemoglobin digestion markedly antagonized the antimalarial activity of the diamidines, indicating that binding to FPIX is crucial for the activity of diamidine drugs. Pentamidine was not accumulated into uninfected erythrocytes. Pentamidine transport into infected cells exhibits an initial rapid phase, nonsaturable in the micromolar range and sensitive to inhibition by furosemide and glibenclamide. Changing the counter-ion in the order $Cl^- < Br^- < NO_2^- < I^- < SCN^-$ markedly stimulated pentamidine transport. These data suggest that pentamidine is transported although a pore or ion channel with properties similar to those of the recently characterized 'induced permeability pathway' on the infected red cell membrane. In summary, the diamidines exhibit two levels of selectivity against *P. falciparum.* The route of entry and molecular target are both specific to malaria-infected cells and are distinct from targets in other protozoa. Drugs that target the hemoglobin degradation pathway of malaria parasites have a proven record of accomplishment. The employment of induced permeability pathways to access this target represents a novel approach to antiparasite chemotherapy and offers an additional level of selectivity.

The evolution of drug resistance poses significant problems for the treatment of malaria. The widespread failure of many antimalarial agents, chloroquine in particular, highlights the urgent need for new drugs (Foley and Tilley, 1998). Pentamidine and other diamidine compounds have a long history in the treatment of human protozoal infections (Ormerod, 1967). Although preliminary studies have shown that diamidine compounds have activity against *P. falciparum* (Bell et al., 1990), they have never been used to treat malaria.

Because pentamidine is poorly membrane-permeable, its selective activity against protozoan parasites is attributed to parasite-specific uptake mechanisms. Active transporters have been described in *Leishmania* species and trypanosomes that allow pentamidine to accumulate to high concentrations (Carter et al., 1995; de Koning and Jarvis, 1999). After accumulation within these parasites, pentamidine is thought to attack a number of biological targets (Basselin et al., 1996; Morty et al., 1998; Reddy et al., 1999).

We assume that pentamidine operates at an intracellular site in *Plasmodium falciparum*. How this highly charged, water-soluble drug manages to penetrate the plasma membrane of the infected red blood cell is unknown, as is the mechanism by which it kills the parasite after accumulation.

In this report, we address both these issues. We demonstrate specific and extensive accumulation of pentamidine into erythrocytes infected with *P. falciparum.* Kinetic and pharmacological characterization of the initial phase of drug uptake indicates that the drug penetrates the infected cell through a parasite specific pore with properties similar to those of the new permeability pathways (NPP) induced by the parasite on the surface of the infected erythrocyte (Ginsburg et al., 1983; Ginsburg and Stein, 1988; Kirk et al., 1994; Upston and Gero, 1995).

Simple penetration through a pore cannot explain the extensive concentration of the drug by the infected cell. We demonstrate that, after penetration through the NPP, the accumulation of pentamidine is largely driven by its binding to ferriprotoporphyrin IX (FPIX) generated during the digestion of hemoglobin (Francis et al., 1997). Malaria parasites normally crystallize toxic FPIX into nontoxic hemozoin (ma-

This work was supported by Wellcome Trust grants to P.G.B. and S.A.W.

ABBREVIATIONS: NPP, new permeability pathway; FPIX, ferriprotoporphyrin IX; PIX, protoporphyrin IX; MD, molecular dynamics.

larial pigment). We demonstrate that the diamidines inhibit the formation of hemozoin from FPIX by interacting directly with FPIX. Furthermore, we show that this interaction is primarily responsible for the antimalarial activity.

Here we explain the antimalarial action of diamidine drugs, based on selective transport through a parasite specific pore and subsequent binding to FPIX. This unique approach to highly selective antimalarial action is based on the targeted exploitation of two parasite-specific pathways. Diamidines such as pentamidine represent a very promising starting point for the design of a new class of antimalarial agent.

Materials and Methods

Reagents. All reagents were obtained from Sigma unless otherwise stated. Silicon oil 550 was obtained from Dow Corning limited. [^{3}H]Pentamidine isethionate [specific activity, 3.63 TBq/mmol (98 Ci/mmol), 5 mCi/ml in ethanol; 99.9% pure by high-performance liquid chromatography], unlabeled stilbamidine, propamidine, and berenil were kindly donated by Prof Mike Barrett, Division of Infection of Immunity, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow, UK. The [^{3}H]pentamidine isethionate was originally custom synthesized and purified by Amersham Pharmacia Biotech UK, Ltd. (Little Chalfont, Buckinghamshire, UK).

Culture of *P. falciparum* and Drug Sensitivity Assays. The 3D7, HB3,and K1 strains of *P. falciparum* used were obtained from Prof. D. Walliker, Edinburgh University (Edinburgh, UK) and recloned in house. The TM6 strain was obtained from Dr. P. Tan-Ariya (Mahidol University, Bangkok, Thailand). The chloroquine resistant isolates K1 and TM6 originate from Thailand and have recently been fully characterized in terms of the chloroquine phenotype and genotype (Mungthin et al., 1999). Parasites were maintained in continuous culture and synchronized using standard techniques. When free parasites were required, they were released from the host cell using the method of Elford (1993). The sensitivity of the infected erythrocytes to various drugs was determined by measuring the ability of serial dilutions of drugs to inhibit the incorporation of radiolabeled [^{3}H]hypoxanthine into parasite nucleic acids (Desjardins et al., 1979). In each case, the lowest feasible inoculum size was used (0.5% parasitemia, 1% hematocrit). IC_{50} values were calculated for each assay using the four-parameter logistic method (Grafit program; Erithacus Software, Kent, UK). The effect of the combination of diamidines and Ro 40-4388 on parasite growth was tested by titration of the two drugs at fixed ratios proportional to their IC_{50} values. The fractional inhibitory concentrations of the resulting IC_{50} values were plotted as isobolograms (Berenbaum, 1978).

The Effect of Furosemide on the in Vitro Activity of Pentamidine. Synchronous trophozoite stage (approximately 36 h) cultures were washed twice in growth medium without serum. Cultures were suspended at an inoculum size of 1 (inoculum size = percent parasitemia × percent hematocrit) in growth medium without serum, containing the following concentrations of pentamidine; 0, 10, 30, 100, 300, 1,000, 3,000, and 10,000 nM. Parallel cultures contained the same concentrations of pentamidine plus 100 μM furosemide, which was diluted into prewarmed medium (37°C) from a 10 mM stock in dimethyl sulfoxide. After incubation for 6 h at 37°C, the cultures were washed three times in complete growth medium to remove unbound drug. The cells were then resuspended in complete growth medium at an inoculum size of 1. [^{3}H]Isoleucine was added (1 μCi/ml) and the cultures were gassed and returned to the incubator for a further 24 h. After incubation, the cells were pelleted and exposed to 0.15% saponin at room temperature for 10 min to lyse the host cells and uninfected erythrocytes. Free parasites were pelleted, the supernatant was discarded, and the cell samples were taken and processed for scintillation counting. Growth was assessed in the absence of pentamidine and in the presence or absence of 100 μM furosemide, as appropriate. For each pentamidine concentration in the presence or absence of 100 μM furosemide, the results were expressed as percentage of control growth in the absence of pentamidine.

Measurement of the Uptake of [^{3}H]Pentamidine. Uninfected erythrocytes or erythrocytes infected with synchronized trophozoites of *Plasmodium falciparum* were suspended in the appropriate buffer containing [^{3}H]pentamidine at a concentration of 50 nM. Preliminary experiments established that there was no significant difference in pentamidine accumulation of the different clones.

For the initial rate of uptake studies, the infected cells were enriched to approximately 70% parasitemia using Plasmagel. The cells were suspended in HEPES-buffered RPMI medium without bicarbonate, pH 7.4. The suspension (typically 10^8 cells/ml) was prewarmed to 37°C in a water bath. The pentamidine and furosemide were added at time 0 (the final pentamidine concentration was adjusted to 20 μM using unlabeled drug). At the required times, 200-μl samples were removed and transferred to chilled microcentrifuge tubes containing 800 μl of ice-cold RPMI, layered over 400 μl of silicon oil followed by immediate centrifugation (15,000*g* for 2 min). The tube tip containing the cell pellet was cut off and the cells were lysed with 100 μl of distilled water. The lysate was solubilized and decolorized by adding 100 μl of a cocktail containing five parts quaternary ammonium hydroxide, two parts H_2O_2, and two parts glacial acetic acid. The samples were then counted by liquid scintillation counting. Subsequent influx experiments to test the effect of counter-ion substitution (buffers as used by Kirk and Horner, 1995b) and to test the concentration-dependence of furosemide were performed at a single time point of 1 min. Initial surface binding of [^{3}H]pentamidine was assessed by fast diluting labeled pentamidine into ice-cold cell suspensions and centrifuging immediately. Counts attributable to surface binding were subtracted from the pellet counts. For the remainder of the accumulation experiments, reactions were terminated by layering 1 ml of the cell suspension over 400 μl of silicon oil and centrifuging and processing as described above. Binding was measured after 2 h in 50 nM [^{3}H]pentamidine and various concentrations of unlabeled pentamidine, in the presence or absence of 10 μM Ro 40-4388.

Nonspecific uptake was determined by measuring and subtracting the counts attributable to an equal number of uninfected erythrocytes. The cellular accumulation ratio is the ratio of the amount of pentamidine in the infected cell to the amount of pentamidine in a similar volume of buffer after incubation. The volume of the infected cell was assumed to be the same as that of the uninfected cell (Kirk et al., 1994). The intracellular drug concentration was calculated by multiplying the cellular accumulation ratio by the buffer drug concentration after incubation.

UV/Visible Spectral Scans. FPIX or protoporphyrin IX (PIX) (Porphyrin Products) solutions were prepared fresh for each experiment. Solutions (3 mM) were prepared in 0.1 M NaOH. Immediately before each scan, samples were fast diluted into 0.2 M HEPES buffer, pH 7.0, to give a final concentration of 3 μM. in the presence or absence of 3 μM pentamidine. Samples (400 μl) were placed in quartz cuvettes and scanned against the appropriate blank in a Hewlett Packard 8452A diode array spectrophotometer (Hewlett Packard, Palo Alto, CA).

Binding of Pentamidine to FPIX or PIX in Vitro. The binding of [^{3}H]pentamidine to FPIX or PIX adsorbed to erythrocyte ghost membranes was measured as described previously for [^{3}H]chloroquine (Bray et al., 1999)

Determination of the Effect of Diamidines and Chloroquine on the Production of Hemozoin in Vitro. Assays were performed as described in Bray et al. (1999). Briefly, an aliquot of trophozoite lysate (100 μl) and FPIX (100 μl of 3 mM in 0.1 M NaOH) were mixed with an aliquot of 1 M HCl (10 μl) and sodium acetate (500 mM, pH 5.2) was added to give a volume of 900 μl in each tube. A series of drug concentrations were prepared in water and 100 μl of each added to the appropriate samples. Samples were mixed and

incubated for 12 h at 37°C, with occasional mixing. After incubation, samples were centrifuged (15,000 g, 15 min, 21°C) and the hemozoin pellet repeatedly washed with 2% w/v SDS in 0.1 M sodium bicarbonate, pH 9.0, with sonication (30 min, 21°C, bath sonicator; Decon FS100 Ultrasonics Ltd, UK) until the supernatant was clear (usually 3–4 times). After the final wash, the supernatant was removed and the pellet was resuspended in 1 ml of 0.1 M NaOH and incubated for a further 1 h at room temperature. Samples were then mixed by aspiration with a pipette. The hemozoin content was determining by measuring the absorbance at 400 nm (DU640 spectrophotometer; Beckman Coulter, Fullerton, CA) using a 1-cm quartz cuvette. The amount of hemozoin formed during the incubation was corrected for preformed hemozoin (the amount of preformed hemozoin in the parasite extract was determined from a sample containing extract, but no substrate, which was incubated and repeatedly washed with 2% SDS as stated previously). The concentration of drug required to produce 50% inhibition of hemozoin production (IC_{50}) was determined graphically as described for the drug sensitivity assays.

Molecular Modeling Studies. All molecular modeling was carried out on an O2 R5000 silicon graphics workstation within the Cerius2 molecular modeling environment. Simulation studies were carried out on pentamidine and heme. Each molecule was energy minimized from the 'as constructed' conformation using the universal force field. The universal force field is able to paramatize a wider range of atom types than earlier force fields.

Energy minimization alone is able to find only the nearest energy minimum to the starting conformation of a given system. To generate a wider representative set, we have employed the method of simulated annealing similar to that employed by Milne (1997). Molecular dynamics (MD) runs at 298K for a simulation time of 5 ps were performed for each drug molecule, and the resulting structure was energy minimized to gain a low-energy conformation. This process was repeated until 10 structures per molecule had been generated. For each MD calculation, the last minimized structure in the set was used as the starting conformation for the next MD simulation.

The aims of the modeling study were to investigate the potential of pentamidine for interacting with heme, the proposed drug receptor site within the parasite food vacuole. Having generated a set of conformations for pentamidine, an investigation into its interaction with haem was carried out. A model of heme (ferriprotoporphyrin IX) was constructed with a single hydroxyl group ligated to the Fe atom at an axial coordination site above the ring. This unit was then energy minimized with the Universal force field potential set. From earlier studies, it was found that the conformation in which both carboxylic acid groups were orientated on the same side of the porphyrin ring (α-hematin) was lower in energy that when the groups were on opposite faces (β-hematin).

For the pentamidine model obtained after energy minimization-dynamics simulation, the lowest energy (folded) conformation was chosen to generate a complex with haem in the following manner. We have found, from previous studies, that antimalarial agents containing aromatic rings can π-stack with the delocalized π-system of the porphyrin ring. With this restriction, the porphyrin model was intercalated between the two aromatic rings of pentamidine in such a way that both benzene rings could potentially form a π-stacking interaction with the planar haem ring. From this starting point, the dimers were then energy minimized using the universal parameter set.

Results

In Vitro Activity of Diamidine Compounds. The diamidine compounds were tested in vitro against a panel of *P. falciparum* clones that have varying susceptibility to the standard antimalarial drugs chloroquine, quinine, and pyrimethamine (Table 1).

As a group, the diamidines were found to exhibit promising in vitro antimalarial activity, comparable with that of the standard antimalarial agents (Table 1). The rank-order of efficacy was: propamidine stilbamidine pentamidine berenil. Importantly, no cross-resistance was found: if anything, chloroquine-resistant clones (K1 and TM6) appear to be slightly more susceptible to the diamidines than chloroquine-susceptible clones (HB3 and 3D7).

Pentamidine Is Transported through the NPP. In order to probe the antimalarial mode of action of the diamidines, the uptake of radiolabeled pentamidine was investigated

Pentamidine uptake into erythrocytes infected with *Plasmodium falciparum* displays two distinct phases: a period of rapid uptake is observed over the first 5 min, followed by a slower phase of drug accumulation lasting several hours (Fig. 1A). After 3 h, pentamidine accumulation is extensive, reaching levels 500-fold greater than the incubation medium. Pentamidine is not significantly accumulated by uninfected erythrocytes, indicating that the pathways responsible for drug accumulation are parasite-specific.

Both phases of drug accumulation were investigated. The rapid initial phase of pentamidine uptake is nonsaturable in the micromolar range and is effectively inhibited by furosemide at 100 μM (Fig. 1B). At this concentration, furosemide is known to inhibit the transport of substrates through the new permeability pathway (NPP) that is induced in the host erythrocyte membrane by the intracellular parasite (Kirk et al., 1994; Upston and Gero, 1995). Furthermore, the IC_{50} value of inhibition of pentamidine uptake (approximately 10 μM) is similar to the IC_{50} value for the inhibition of transport of known NPP substrates by furosemide (Kirk and Horner, 1995b)

Pentamidine uptake is also blocked by other NPP inhibitors, such as glibenclamide, phloridzin, and NPPB (Kirk et al., 1993; Kirk and Horner, 1995a; Upston and Gero, 1995; data not shown). By contrast, pentamidine uptake is not

TABLE 1
Sensitivity of *P. falciparum* clones to standard antimalarial agents and to diamidines

Drug	Strain			
	HB3 (CQS)	3D7 (CQS)	K1 (CQR)	TM6 (CQR)
Chloroquine	12 ± 2.1	14 ± 2.9	222 ± 37	167 ± 27
Quinine	65 ± 15	35 ± 12	176 ± 15	199 ± 20
Pyrimethamine	790 ± 187	6.8 ± 1.7	6307 ± 815	N.D.
Pentamidine	126 ± 56	88 ± 56	66 ± 15	65 ± 19
Propamidine	10.8 ± 2.1	43.2 ± 6.6	5.6 ± 1.2	6.5 ± 2.8
Stilbamidine	39 ± 6.9	N.D.	27 ± 9	N.D.
Berenil	N.D.	831 ± 284	185 ± 99	236 ± 57

N.D., not determined.

inhibited significantly by millimolar concentrations of arginine, adenine, inosine, putrescine, or spermidine (data not shown). These compounds are inhibitors of pentamidine transport systems known to be present in *Leishmania* species and trypanosomes (Carter et al., 1995; Basselin et al., 1996; de Koning and Jarvis, 1999). Transport of pentamidine into isolated parasites seems to be much less sensitive to furosemide compared with intact infected cells (IC_{50} values of 300 μM versus 10 μM; Fig. 2). These data suggest that the furosemide-sensitive pathway is on the host erythrocyte membrane, consistent with the location of the NPP.

Substituting chloride in the bathing medium with other anions is known to stimulate the transport of cationic substrates by the NPP (Kirk and Horner, 1995b). Similarly, we show that substituting chloride with thiocyanate, nitrate, bromide, or iodide results in a marked stimulation of pentamidine uptake (Fig. 3).

Pentamidine is known to inhibit several transport systems on the surface of eukaryotic cells (Kandpal et al., 1995; Kleyman et al., 1995; Navas et al., 1996; de Koning and Jarvis, 1999). It is possible that pentamidine may exert its antimalarial effect by inhibiting the transport of essential nutrients or metabolites across the host erythrocyte membrane. In this case, the extensive uptake of pentamidine by the infected cell may have little relevance in the mode of action of the drug. However, coincubation with furosemide significantly protects the parasite from the antimalarial effect of pentamidine, increasing the pentamidine IC_{50} value by at least an order of magnitude (Fig. 4). These data suggest that the extensive uptake of pentamidine through the furosemide-sensitive pathway is required for antimalarial activity.

Pentamidine Binds to FPIX in the Malaria-Infected Erythrocyte and Inhibits the Crystallization of FPIX into Hemozoin. Pentamidine accumulation over the second slower phase is saturable Scatchard analysis (Fig. 5A) reveals at least two classes of binding site in malaria-infected erythrocytes that exhibit Michael-Menten kinetics: a high affinity site (K_d value of 2.5 μM) and a lower affinity site (K_d value of approximately 200 μM). These data indicate that malaria parasites possess saturable intracellular receptors for pentamidine. Malaria parasites continuously generate FPIX in large quantities from the action of parasite proteases on host cell hemoglobin and FPIX is known to act as a

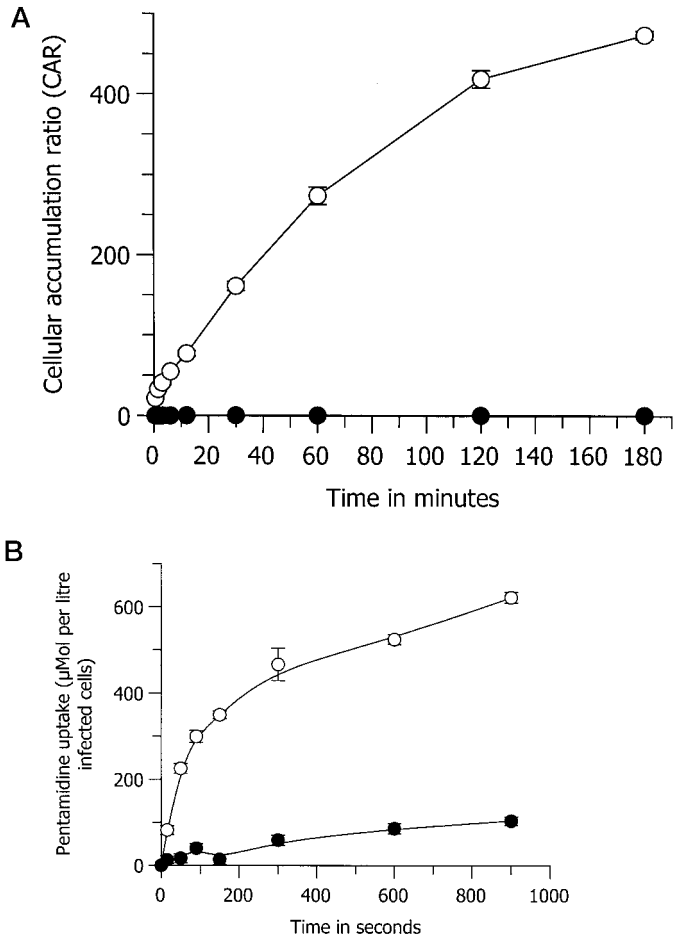

Fig. 1. A, time course of uptake of 50 nM [^{3}H]pentamidine into uninfected erythrocytes (●) and erythrocytes infected with the HB3 strain of *P. falciparum* (○). Data are means ± S.E. from six individual experiments. B, time course of the initial uptake of 20 μM [^{3}H]pentamidine into HB3 infected erythrocytes in the presence (●) or absence (○) of 100 μM furosemide. Data are means ± S.E. from six individual experiments.

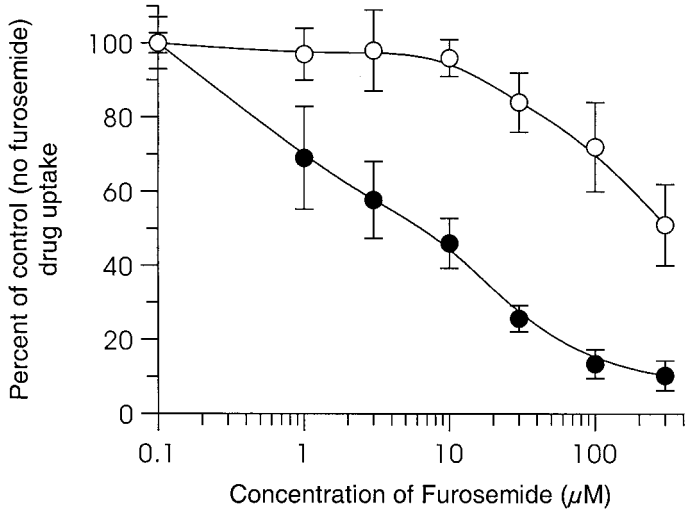

Fig. 2. Concentration-dependent inhibition of [^{3}H]pentamidine uptake by furosemide. Data are presented for intact erythrocytes (●) infected with the HB3 strain of *P. falciparum* and for isolated parasites of the same strain (open symbols). Data are means ± S.E. from five individual experiments.

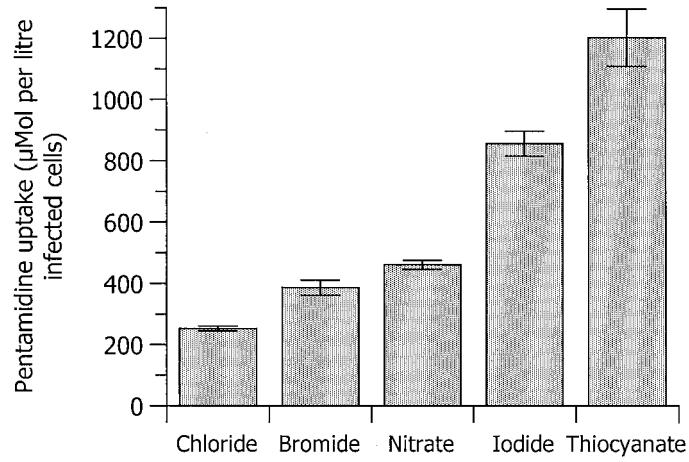

Fig. 3. The effect of substituting other permeant anions for chloride on the initial uptake of 20 μM [^{3}H]pentamidine into erythrocytes infected with the K1 strain. Data represent means ± S.E. for 10 individual experiments.

receptor for several antimalarial drugs (Francis et al., 1997; Bray et al., 1998, 1999; Mungthin et al., 1998). Pentamidine has structural features that suggest it may bind to FPIX (see below).

Indeed, pentamidine does bind to FPIX in vitro: data presented in Fig. 5B show a pronounced quenching of the Soret peak of FPIX in the presence of a molar equivalent of pentamidine. This is confirmed by in vitro binding data obtained using radiolabeled drug. These data show that pentamidine binds to FPIX adsorbed to erythrocyte ghost membranes with an affinity (K_d) value of 2.9 μM (Fig. 5C).

This value is remarkably similar to the K_d value of 2.5 μM observed for high affinity pentamidine binding to intact infected cells, suggesting that binding of pentamidine to FPIX may occur in the infected cell. This is confirmed by using Ro 40-4388, a specific inhibitor of parasite hemoglobinase enzymes (Moon et al., 1997). This inhibitor allows hemoglobin digestion to be blocked reversibly in situ. Consequently, the release of FPIX in is also blocked (Bray et al., 1999). Because FPIX exists only transiently in viable cells, this maneuver permits the measurement of the contribution of FPIX binding in the mechanism of drug accumulation of intact infected erythrocytes (Bray et al., 1998, 1999; Mungthin et al., 1998). A 10 μM concentration of Ro 40-4388 is sufficient to block the generation of FPIX by approximately 70% (Bray et al., 1999). Under the same conditions, the high-affinity accumulation of pentamidine is reduced by 60% (Fig. 6).

Our strategy reduces the number of FPIX molecules but does not alter their nature. This would be expected to reduce the drug binding capacity but not alter the binding affinity. This is exactly what is observed experimentally, providing compelling evidence that binding to FPIX drives high-affinity pentamidine accumulation in the parasite. This binding is crucial for the antimalarial activity of all the diamidines: isobole analysis reveals a marked antagonism of the antimalarial activity of pentamidine, propamidine, stilbamidine, or berenil, when combined with Ro 40-4388 (Fig. 7).

Other antimalarial drugs that bind to FPIX are thought to kill parasites by preventing the formation of hemozoin crystals, allowing toxic FPIX to accumulate and ultimately kill the parasite (Slater, 1993; Pagola et al., 2000). Chloroquine is a good example of a drug that works in this way. All of the diamidines are able to inhibit the formation of hemozoin in vitro at concentrations comparable with chloroquine (Fig. 8). Taken together, all of our data provide good evidence that the diamidines kill parasites by binding avidly to FPIX, inhibiting the formation of hemozoin and causing a build-up of toxic FPIX or FPIX-drug complex.

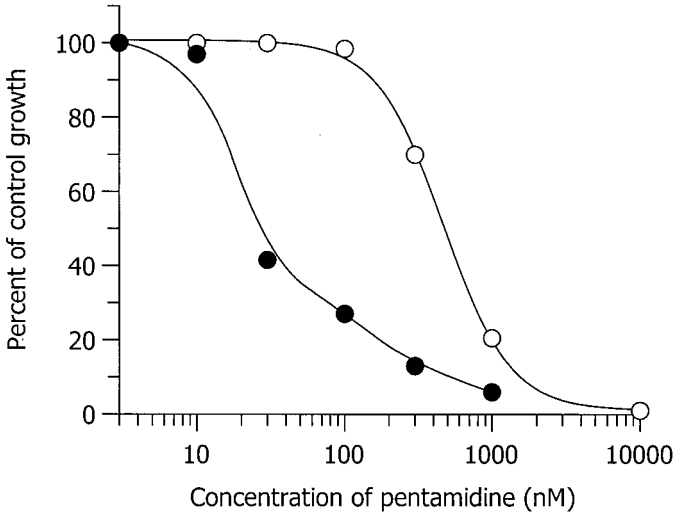

Fig. 4. The effect of the presence (○) or absence (●) of 100 μM furosemide on the sensitivity of erythrocytes infected with the HB3 strain of *P. falciparum* to pentamidine. Data are means of two individual experiments each comprising five observations.

Molecular Modeling of Pentamidine and Its Interaction with FPIX. Energy-minimized structures generated by the molecular modeling program are presented in Fig. 9, A and B. Rather than adopting a linear conformation, the intramolecular interactions seem to colocalize the two charged groups of pentamidine (Fig. 9A).

The resulting alteration of the electrical potential of the molecular surface may facilitate the transport of pentamidine through the NPP: if the barrel of the NPP contains positively charged residues as hypothesized by Kirk and Horner (1995b), the potential repulsive effects of the charged region presumably would be less severe than if the two charged groups were at either end of the molecule. Alternatively, this conformation may favor ion pairing with the permeant anion. At a maximum of just over 6 Å in diameter, the molecule is comfortably within the 10–12 Å upper size limit proposed for substrates of the NPP (Staines et al., 2000).

This conformation of pentamidine also facilitates a proposed interaction with FPIX, in which an aromatic phenyl ring of the pentamidine molecule π stacks with the aromatic porphyrin ring (Fig. 9B). Other antimalarial drugs that disrupt the formation of hemozoin also pi stack with FPIX in this manner. The drugs are thought to sterically hinder the formation of a bond between the iron of one porphyrin and a carboxylate group of an adjacent porphyrin (O'Neill et al., 1997). This bond is required for the formation of the hemozoin crystal (Pagola et al., 2000). In the configuration depicted in Fig. 5C, the phenyl ring of pentamidine is positioned optimally for π stacking (approximately 3.5 Å from the face of the porphyrin ring). Consequently, the amidine nitrogens are greater than 3.5 Å away from the porphyrin iron. At this distance, an electrostatic bond is not favored. This is supported by the demonstration that strong drug binding also takes place when the iron is removed from FPIX. Pentamidine also quenches the Soret peak of protoporphyrin IX (PIX) (Fig. 10). Furthermore, the binding of pentamidine to PIX adsorbed to erythrocyte ghost membranes displays an affinity very similar to the binding of pentamidine to FPIX in the same system (Fig. 5C).

Discussion

Here we demonstrate potent in vitro antimalarial activity of several diamidine compounds. The activity of the diamidines against *P. falciparum* compares favorably and without cross-resistance with that of the standard antimalarial agents chloroquine, quinine, and pyrimethamine (Table 1). The antimalarial selectivity of these compounds can be attributed to the targeting of at least two distinct parasite-specific pathways.

Transport of Diamidines into the Malaria-infected Erythrocyte. We have shown that pentamidine is essentially impermeable to normal uninfected erythrocytes but enters malaria infected erythrocytes rapidly, via a furo-

semide-sensitive and nonsaturable pathway (Fig. 1, A and B). This pathway shows a striking similarity to the well-characterized NPP induced by the intracellular parasite in the host erythrocyte membrane.

The membrane of the human erythrocyte normally has very low cation permeability. However, after infection with *P. falciparum*, the permeability to K^+ and several organic cations is markedly increased (Kirk and Horner, 1995b; Staines et al., 2000). The NPP are anion-selective; like a number of other anion-selective channels (Franciolini and Petris, 1990), they are known to have significant permeability to cations (Kirk and Horner, 1995b).

The transport of cations through the NPP is stimulated by the substitution of Cl^- with other counter-ions (Kirk and Horner, 1995b). Similarly, we demonstrate that the transport of pentamidine into malaria-infected erythrocytes is

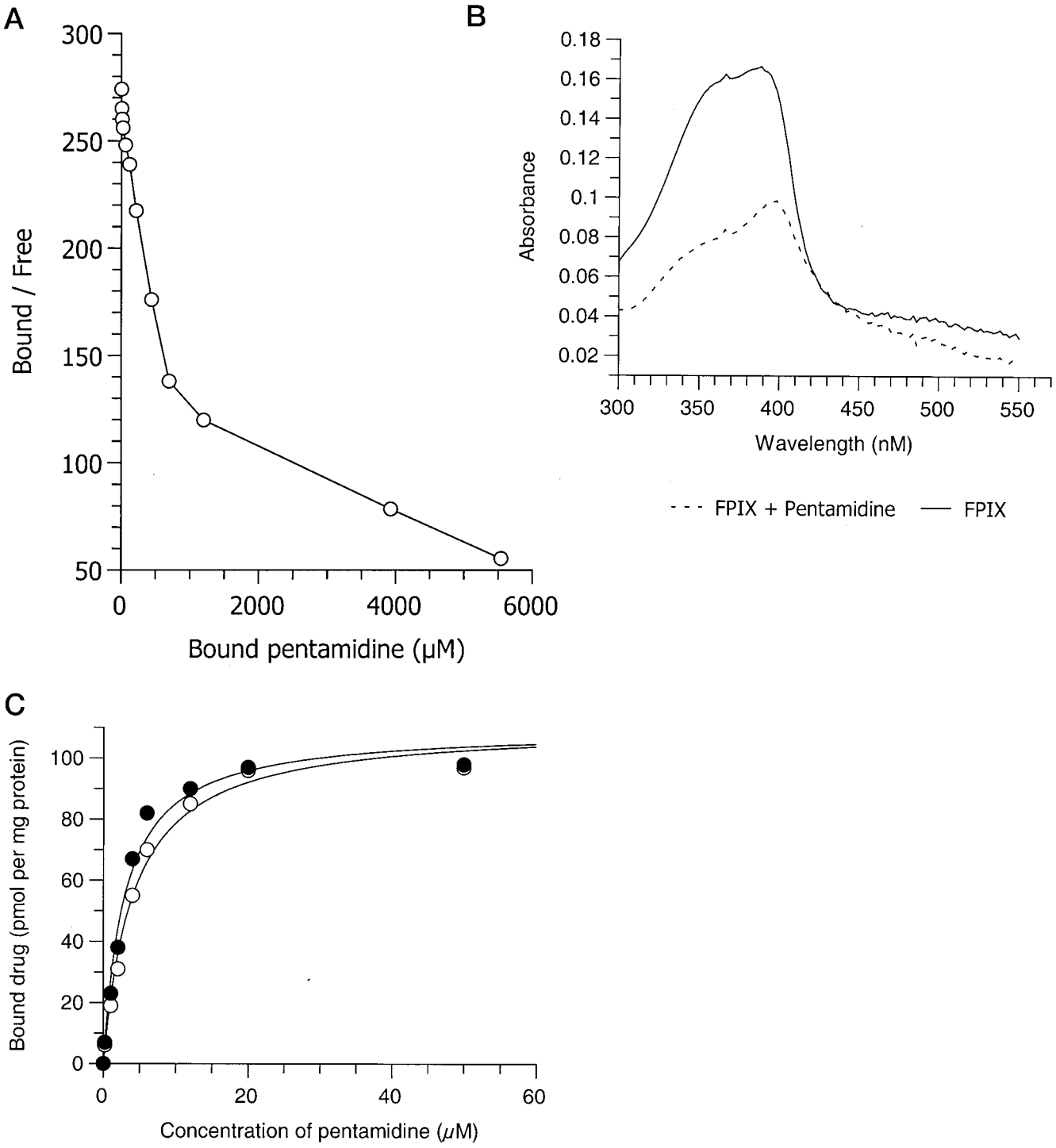

Fig. 5. A, Scatchard plot of [^{3}H]pentamidine binding in intact erythrocytes infected with the HB3 strain of *P. falciparum*, measured after 2 h exposure. B, UV/visible spectral scan of 3 μM FPIX in the presence or absence of 3 μM pentamidine. C, binding of [^{3}H]pentamidine to FPIX (●) and PIX (○) adsorbed to erythrocyte ghost membrane preparations. Data are means ± S.E. from 10 individual experiments.

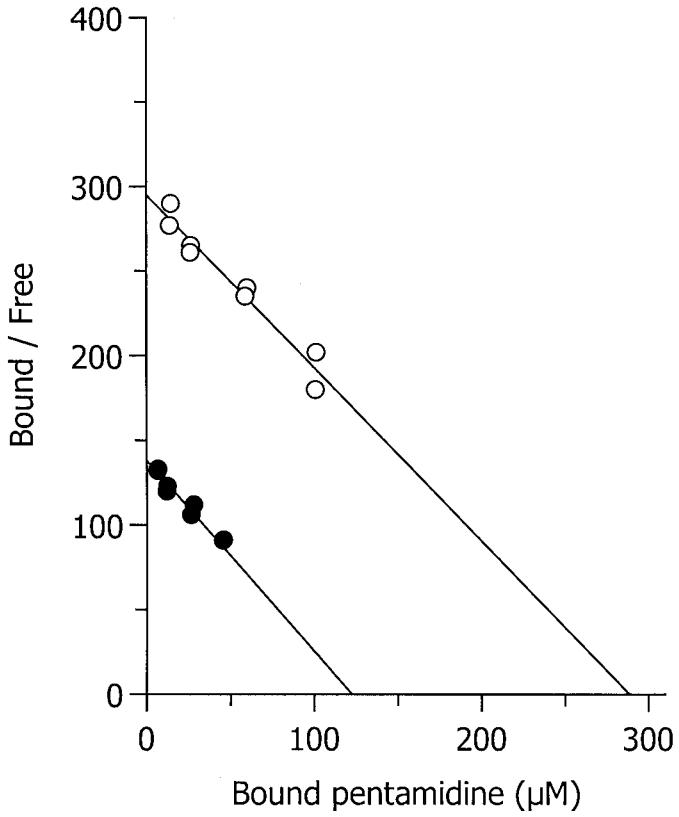

Fig. 6. Scatchard plot of high-affinity [^{3}H]pentamidine binding to intact infected erythrocytes (HB3 strain) in the presence (●) or absence (○) of 10 μM Ro 40-4388.

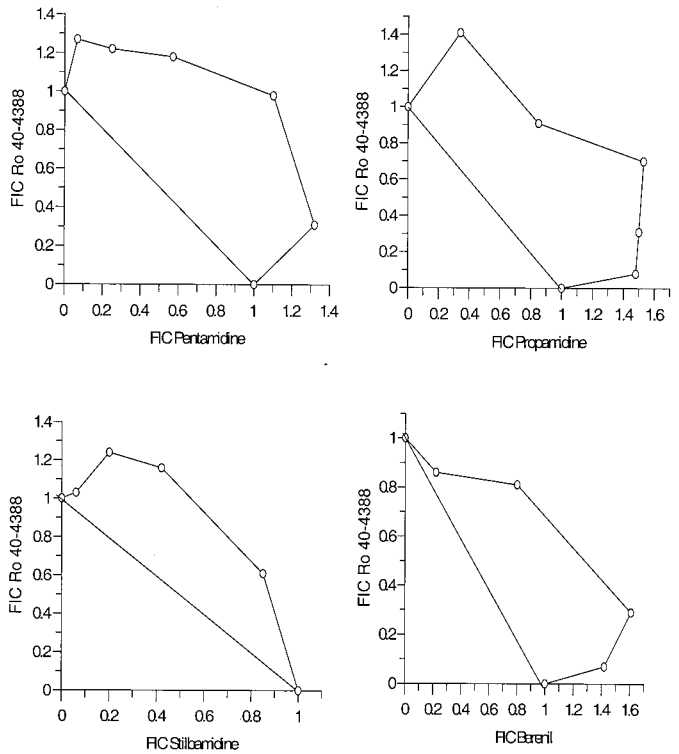

Fig. 7. Isobole plots of diamidine compounds in combination with the protease inhibitor Ro 40-4388 (HB3 strain).

markedly stimulated by the substitution of Cl^- with SCN^-, NO_3^-, Br^-, or I^- (Fig. 3).

This phenomenon seems to operate at the level of the host cell membrane or the parasitophorous vacuole membrane, because the transport of pentamidine into free parasites is not influenced significantly by counter-ion substitution (data not shown). Furthermore, the transport of pentamidine into free parasites is much less sensitive to inhibition by furosemide, with an IC_{50} value of 300 μM versus 10 μM for intact infected erythrocytes (Fig. 2). Taken together, our data suggest that pentamidine can gain access to the intracellular parasite via a pathway exhibiting all of the functional characteristics of the NPP.

The Inhibition of FPIX Crystallization by Diamidine Compounds. Pentamidine is concentrated by the infected cells 500-fold within 3 h (Fig. 1A). Pore systems such as the NPP will allow the equilibration of substrates across a membrane barrier but will not themselves allow the substrate to be concentrated. Therefore, additional factors such as intracellular drug binding or parasite active transporters are required to concentrate the drug. One potential parasite drug receptor that is present in the appropriate quantity is FPIX. The ability of the diamidines to quench the Soret peak of FPIX shows that a binding interaction takes place in vitro (Fig. 5B). Energy minimization modeling indicates that the most stable conformation effectively intercalates the porphyrin within the diamidine molecule, with the aromatic phenyl ring of the amidine group π stacking on the surface of the porphyrin ring (Fig. 9B). In this conformation, the amidine nitrogens are unlikely to coordinate with the iron of the porphyrin. In support of our model, we provide strong evidence that the porphyrin iron is not required for pentamidine binding.

Several lines of evidence suggest that pentamidine binds to FPIX inside the parasite and that this binding makes a significant contribution to the amount of drug accumulated. Scatchard analysis of pentamidine accumulation kinetics after 2 h reveals the existence of at least two saturable binding sites (Fig. 5A). The high-affinity binding site displays an almost identical affinity for pentamidine, as does FPIX in

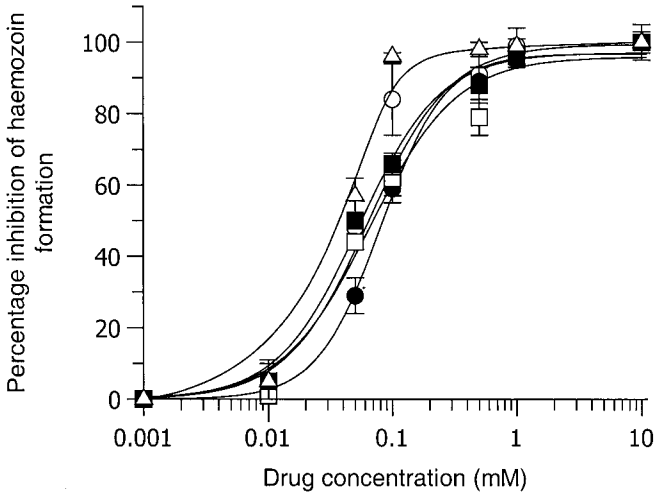

Fig. 8. Comparison of the ability of chloroquine and diamidine compounds to inhibit the formation of hemozoin from FPIX in vitro. Compounds are: pentamidine (○), berenil (●), propamidine (□), stilbamidine (■) and chloroquine (△). Data are means ± S.E. from five individual experiments.

vitro (Fig. 5C). Furthermore, inhibition of hemoglobin digestion and FPIX release by the specific parasite hemoglobinase inhibitor Ro 40-4388, effectively inhibits the high affinity accumulation of pentamidine into intact infected cells (Fig. 6).

Finally, it is clear that the binding of diamidines to FPIX is fundamentally important for antimalarial activity. Isobole analysis reveals a marked antagonism between all of the diamidines and Ro 40-4388 (Fig. 7). Other FPIX-binding antimalarial drugs such as chloroquine are thought to kill parasites by inhibiting the formation of hemozoin, allowing FPIX to build up to toxic levels and overwhelm the parasite (Slater, 1993; Foley and Tilley, 1998). All of the diamidines were able to inhibit the crystallization of FPIX in vitro and with similar efficiency to chloroquine (Fig. 8). Importantly, inhibition was observed at micromolar concentrations that

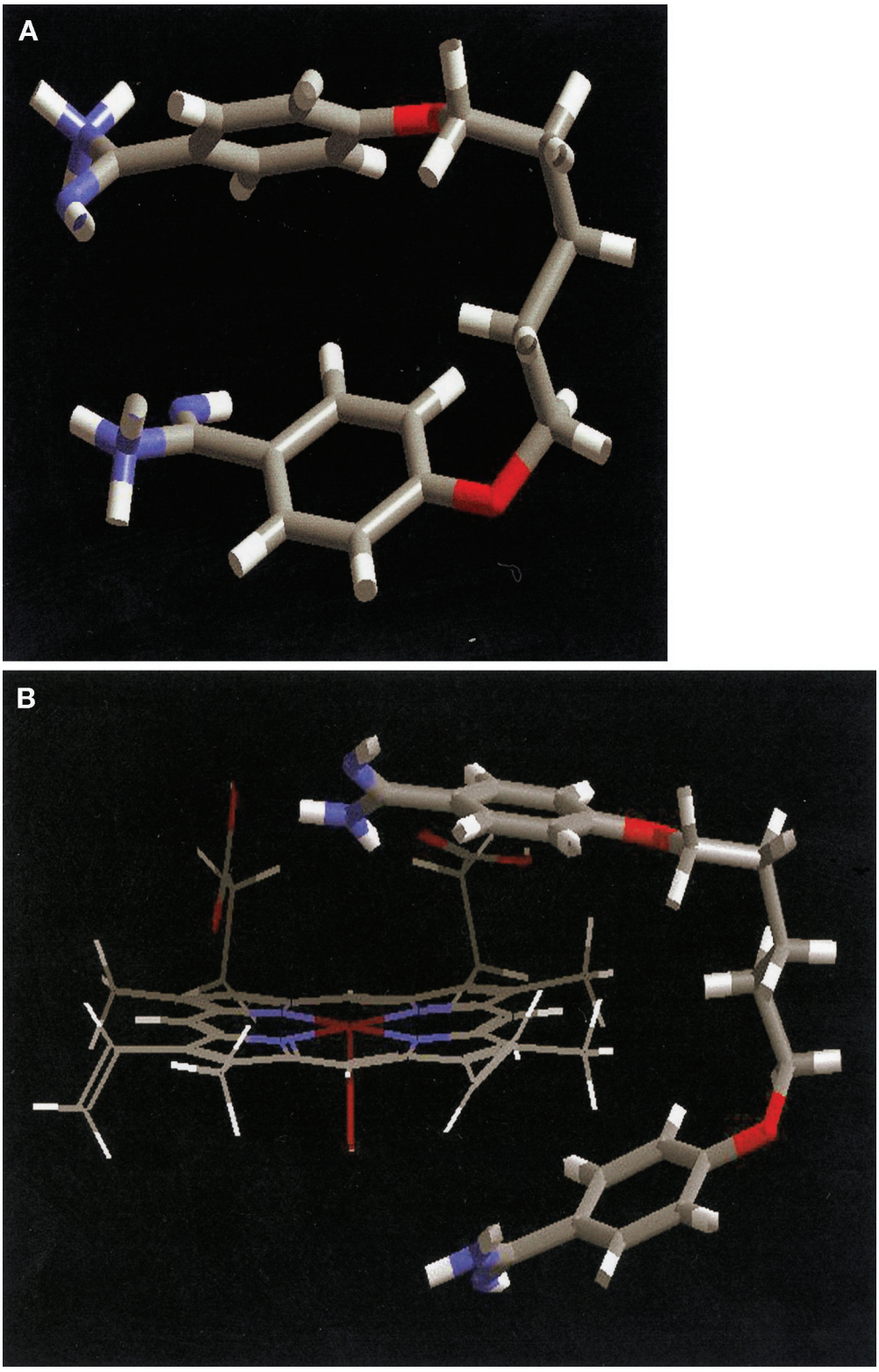

Fig. 9. A, energy-minimized representation of pentamidine. B, energy-minimized representation of the association of pentamidine with FPIX.

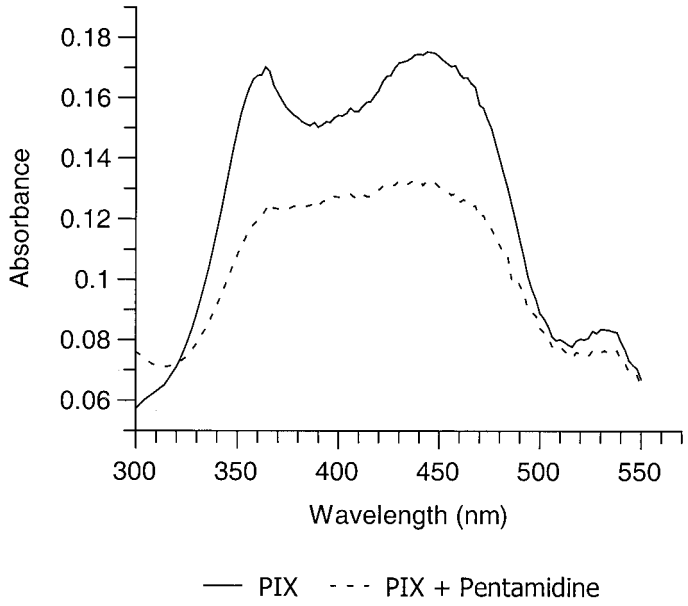

Fig. 10. UV/visible spectral scan of 3 μM PIX in the presence or absence of 3 μM pentamidine.

are easily achieved inside the parasite within 3 h (Fig. 1A). These data reveal that the interaction between FPIX and the diamidine drugs is a critical event in parasite killing and pinpoint FPIX as the likely molecular target of this class of antimalarial.

Conclusions

Diamidine drugs are impermeable to normal human erythrocytes but are taken up rapidly into *P. falciparum*-infected erythrocytes via NPP induced by the parasite in the host cell membrane. Once inside the infected cell, the drugs bind avidly to FPIX and kill the parasites, probably by inhibiting FPIX crystallization.

Currently available diamidines are highly toxic and are very poorly absorbed orally. However, recent reports suggest that it may be possible to overcome both of these problems by using relatively simple chemical substitutions (Francesconi et al., 1999). Here we show that diamidines are likely to share a common mode of action with chloroquine but are not recognized by the chloroquine resistance mechanism. Therefore, our studies define a novel antimalarial pharmacophore with considerable advantages over conventional FPIX-binding antimalarial drugs.

Acknowledgments

We would thank M. P. Barrett for the generous gift of radiolabeled pentamidine.

References

Basselin M, Lawrence F and Robert-Gero M (1996) Pentamidine uptake in *Leishmania donovani* and *Leishmania amazonensis* promastigotes and axenic amastigotes. *Biochem J* **315:**631–634.

Bell CA, Hall JE, Kyle DE, Grogl M, Ohemeng KA, Allen MA and Tidwell RR (1990) Structure-activity relationships of analogs of pentamidine against *Plasmodium falciparum* and *Leishmania mexicana amazonensis*. *Antimicrob Agents Chemother* **34:**1381–1386.

Berenbaum MC (1978) A method for testing for synergy with any number of agents. *J Infect Dis* **137:**122–30.

Bray PG, Mungthin M, Ridley RG and Ward SA (1998) Access to hematin: the basis of chloroquine resistance. *Mol Pharmacol* **54:**170–179.

Bray PG, Janneh O, Raynes KJ, Mungthin M, Ginsburg H and Ward SA (1999) Cellular uptake of chloroquine is dependent on binding to ferriprotoporphyrin IX and is independent of NHE activity in *Plasmodium falciparum*. *J Cell Biol* **145:** 363–376.

Carter NS, Berger BJ and Fairlamb AH (1995) Uptake of diamidine drugs by the P2 nucleoside transporter in melarsen-sensitive and -resistant *Trypanosoma brucei brucei*. *J Biol Chem* **270:**28153–28157.

Desjardins RE, Canfield CJ, Haynes JD and Chulay JD (1979) Quantitative assessment of antimalarial activity in vitro by a semiautomated microdilution technique. *Antimicrob Agents Chemother* **16:**710–718.

Elford BC (1993) Generating viable extra-erythrocytic forms of *Plasmodium falciparum*. *TDR News* **41:**11.

Foley M and Tilley L (1998) Quinoline antimalarials: mechanisms of action and resistance and prospects for new agents. *Pharmacol Ther* **79:**55–87.

Francesconi I, Wilson WD, Tanious FA, Hall JE, Bender BC, Tidwell RR, McCurdy D and Boykin DW (1999) 2,4-Diphenyl furan diamidines as novel anti-Pneumocystis carinii pneumonia agents. *J Med Chem* **42:**2260–2265.

Franciolini F and Petris A (1990) Chloride channels of biological membranes. *Biochim Biophys Acta* **1031:**247–259.

Francis SE, Sullivan DJ Jr and Goldberg DE (1997) Hemoglobin metabolism in the malaria parasite *Plasmodium falciparum*. *Annu Rev Microbiol* **51:**97–123.

Ginsburg H, Krugliak M, Eidelman O and Cabantchik ZI (1983) New permeability pathways induced in membranes of *Plasmodium falciparum* infected erythrocytes. *Mol Biochem Parasitol* **8:**177–190.

Ginsburg H and Stein WD (1988) Biophysical analysis of a novel transport pathway induced in red blood cell membranes by the malaria parasite. *Prog Clin Biol Res* **252:**317–322.

Kandpal M, Fouce RB, Pal A, Guru PY and Tekwani BL (1995) Kinetics and molecular characteristics of arginine transport by *Leishmania donovani* promastigotes. *Mol Biochem Parasitol* **71:**193–201.

Kirk K, Horner HA, Spillett DJ and Elford BC (1993) Glibenclamide and meglitinide block the transport of low molecular weight solutes into malaria-infected erythrocytes. *FEBS Lett* **323(1–2):**123–128.

Kirk K, Horner HA, Elford BC, Ellory JC and Newbold CI (1994) Transport of diverse substrates into malaria-infected erythrocytes via a pathway showing functional characteristics of a chloride channel. *J Biol Chem* **269:**3339–3347.

Kirk K and Horner HA (1995a) In search of a selective inhibitor of the induced transport of small solutes in *Plasmodium falciparum*-infected erythrocytes: effects of arylaminobenzoates. *Biochem J* **311:**761–768.

Kirk K and Horner HA (1995b) Novel anion dependence of induced cation transport in malaria-infected erythrocytes. *J Biol Chem* **270:**24270–24275.

Kleyman TR, Roberts C and Ling BN (1995) A mechanism for pentamidine-induced hyperkalemia: inhibition of distal nephron sodium transport. *Ann Intern Med* **122:**103–106.

de Koning HP and Jarvis SM (1999) Adenosine transporters in bloodstream forms of *Trypanosoma brucei brucei*: substrate recognition motifs and affinity for trypanocidal drugs. *Mol Pharmacol* **56:**1162–1170.

Milne GW (1997) Mathematics as a basis for chemistry. *J Chem Inf Comput Sci.* **37:**639–44.

Moon RP, Tyas L, Certa U, Rupp K, Bur D, Jacquet C, Matile H, Loetscher H, Grueninger-Leitch F, Kay J, et al. (1997) Expression and characterisation of plasmepsin I from Plasmodium falciparum. *Eur J Biochem* **244:**552–560.

Morty RE, Troeberg L, Pike RN, Jones R, Nickel P, Lonsdale-Eccles JD and Coetzer T (1998) A trypanosome oligopeptidase as a target for the trypanocidal agents pentamidine, diminazene and suramin. *FEBS Lett* **433:**251–256.

Mungthin M, Bray PG, Ridley RG and Ward SA (1998) Central role of hemoglobin degradation in mechanisms of action of 4-aminoquinolines, quinoline methanols, and phenanthrene methanols. *Antimicrob Agents Chemother* **42:**2973–2977.

Mungthin M, Bray PG and Ward SA (1999) Phenotypic and genotypic characteristics of recently adapted isolates of *Plasmodium falciparum* from Thailand. *Am J Trop Med Hyg.* **60:**469–74.

Navas IM, Garcia-Fernandez AJ, Johnson RA, Reguera RM, Balana-Fouce R and Ordonez D (1996) Structural determinants of putrescine uptake inhibition produced by cationic diamidines in the model of trypanosomatid *Crithidia fasciculata*. *Biol Chem* **377:**833–836.

O'Neill PM, Willock DJ, Hawley SR, Bray PG, Storr RC, Ward SA and Park BK (1997) Synthesis, antimalarial activity, and molecular modeling of tebuquine analogues. *J Med Chem* **40:**437–448.

Ormerod WE (1967) Leishmaniasis. *Trop Dis Bull* **64:**585–590.

Pagola S, Stephens PW, Bohle DS, Kosar AD and Madsen SK (2000) The structure of malaria pigment beta-haematin. *Nature (Lond)* **404:**307–10.

Reddy BS, Sondhi SM and Lown JW (1999) Synthetic DNA minor groove-binding drugs. *Pharmacol Ther* **84:**1–111.

Slater AF (1993) Chloroquine: mechanism of drug action and resistance in *Plasmodium falciparum*. *Pharmacol Ther* **57(2–3):**203–235.

Staines HM, Rae C and Kirk K (2000) Increased permeability of the malaria-infected erythrocyte to organic cations. *Biochim Biophys Acta* **1463:**88–98.

Upston JM and Gero AM (1995) Parasite-induced permeation of nucleosides in *Plasmodium falciparum* malaria. *Biochim Biophys Acta* **1236:**249–258.

Send reprint requests to: Dr. Patrick G. Bray, Department of Pharmacology and Therapeutics, The University of Liverpool, Liverpool L69 3BX, United Kingdom. E-mail: p.g.bray@liv.ac.uk